

THE DESTRUCTION OF RED BLOOD
CORPUSCLES IN EXPERIMENTAL
HEMOLYTIC ANEMIA

BY

CURT WASASTJERNA

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PREFACE

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Helsingfors, March 1951.

Curt Wasastjerna.

CHAPTER I

SURVEY OF THE LITERATURE

THE LIFE-SPAN AND DESTRUCTION OF RED BLOOD CORPUSCLES UNDER NORMAL AND PATHOLOGICAL CONDITIONS

Many works on hemolytic anemias have been published in recent years and their pathogenesis has been to some extent clarified. It therefore seems strange that very little is so far known about the destruction of erythrocytes under normal conditions. We do not know how the destruction is regulated or how it takes place. We know that the erythrocytes may be ingested by cells in the reticuloendothelial organs under certain conditions. We know of pathological conditions accompanied by lysis of erythrocytes in the blood stream. But we still do not know whether the normal destruction takes place through phagocytosis, fragmentation, hemolysis, or some other mechanism.

On the other hand, the question of the life-span of the red corpuscles in man now seems to have been finally solved. A few years ago the different authors, having used a large variety of methods, still reported greatly varying life-spans, i. e. 14 to 200 days. Proceeding by different routes, a life-span of 110 to 130 days for a normal erythrocyte has now been shown (A s h b y 1948). The majority of the methods used are modifications of A s h b y's (1919) method which consists of transfusion of blood of a suitable group and »differential agglutination», i. e. agglutination of either the recipient's or the donor's blood cells; this makes it possible to separately enumerate the blood corpuscles transferred.

The so-called A s h b y method is advantageous in many respects. For instance, it allows us to analyse the hemolytic anemias and other pathological conditions in which the life-span of the corpuscles is less than normal. By transfusing erythrocytes from healthy people to patients with hemolytic anemia and vice versa we can determine whether the shortened life-span of the blood cells is »extracorporeal» or »intracorporeal», in other words, whether it is due to an external factor which destroys the corpuscles or to a defect in them. D a c i e and M o l l i s o n (1943) transfused blood corpuscles from a patient with familial hemolytic jaundice to a healthy person and found that they disappeared from the blood stream in 14 days. When patients with this disease were given blood from healthy people, the donor's erythrocytes lived for a normal period in the recipient's blood. In familial hemolytic

jaundice the cause of the increased destruction is thus probably »intracorpuseular». In the same way Loutit and Mollison (1946) found the cause of acquired hemolytic jaundice to be an »extracorpuseular» factor which also destroys normal corpuscles injected into a patient with this disease.

In the case of the normal destruction of erythrocytes also, the main problem is to find out whether they are so constructed that they live about 120 days and then disappear in one way or another (the longevity theory), or whether there is an »extracorpuseular» factor, i. e. an organ or a substance in the blood which destroys them in approximately 120 days. The Ashby method gives us some information in this respect also. It has been observed that if the number of erythrocytes transferred, per cu. mm. of blood, are enumerated at regular intervals and the results marked on a diagram, the destruction curve will be straight in normal cases (Brown et al. 1944, Callender et al. 1945, Mollison 1947). This shows that the destruction probably depends on the age of the erythrocytes. If an organ, the spleen, for instance, were to destroy the blood cells by chance and independently of their age, the probability of the transfused cells being disintegrated would decrease with their number, and the approach of the curve to the zero-line would be asymptotic. Similarly if a hemolysin were responsible for the destruction, in that a corpuscle would always be disintegrated if it chanced to come into collision with a molecule of the hemolytic agent. An »exponential» destruction curve of this kind has actually been demonstrated by Brown, Hayward, Powell and Wits (1944) and Mollison (1947) in cases of acquired hemolytic jaundice. In spite of the curve being straight in normal cases, the erythrocytes may gradually be influenced by a hemolysin in the blood, but 120 days or more are required for the disintegration of red corpuscles (Ponder 1948), or the corpuscles may be affected to some extent each time they pass through the spleen.

There is a disadvantage in the Ashby method for determining the life-span of the erythrocyte. We cannot prove whether the behaviour of the foreign corpuscles after transfusion is similar to that of the organism's own corpuscles and whether the same rules are followed. The behaviour and the rules are similar, however, which is shown by the same average life-span being obtained by other modern methods (Ashby 1948, Estren and Dameshek 1949). The method of Shemin and Rittenberg (1946) should be mentioned in the first place: blood corpuscles are marked with the nitrogen isotope N_{15} in the amino acid glycine. The average life-span of 127 days is obtained for normal red blood cells.

We thus know something about the general rules of erythrocyte destruction but we do not know, as already stated, in what way the erythrocytes actually disappear from the circulating blood stream.

In the next section of this chapter, the action of various hemolysins on the red corpuscles and some hemolysins that have been found in the organism will be dealt with. Their possible significance for the normal destruction of erythrocytes will also be treated. It has been shown that hemolysins may be the cause of

pathologically increased hemolysis, but it has not been proved that they are, partly or wholly, responsible for the normal erythrocyte destruction.

According to Rous' (1923) survey, fragmentation, caused by mechanical trauma to the circulating corpuscles and phagocytosis of red cells, occurs in normal mammals, whereas it is not certain whether these, or may be a third, unknown process is the main destructive factor. Erythrophagocytosis has been definitely shown in certain conditions, for instance in erythroblastosis foetalis (Senott 1946). It cannot, however, be considered definite that normal erythrocytes in healthy animals and healthy people can be ingested by the cells in the reticulo-endothelial system, since the results of the various authors differ in this respect. Knisely and his co-workers (Knisely 1936, Knisely et al. 1948), in their extensive studies of the blood in the living state, did not find phagocytosis of normal, non-agglutinated corpuscles, but only of those which are coated with protein precipitate and agglutinated (Knisely et al. 1947). On the other hand, MacKenzie, Whipple and Wintersteiner (1941), in similar investigations, observed phagocytosis of normal erythrocytes in the spleen of healthy animals. Ham, Shen, Fleming and Castle (1948) induced fragmentation of red corpuscles in vitro simply by heating the blood. Fragments of corpuscles formed in this way were immediately removed from the circulating blood when previously heated blood was reinjected into experimental animals.

In the majority of works on the destruction of erythrocytes, the spleen has attracted great interest. This is chiefly due to its important role in hemolytic jaundice which is best evidenced by the therapeutical effect of splenectomy in this group of diseases. In familial hemolytic jaundice, the hemolysis decreases after splenectomy although the spherocytosis remains (Meulengracht 1918, Gripwall 1938). Cases of acquired hemolytic jaundice are often completely cured by splenectomy, whereas, in some cases there is an improvement or no response whatsoever (Young 1947, Estren and Dameshek 1949, Evans and Duane 1949). In different kinds of hemolytic jaundice Heilmeyer (1935—1936) and Gripwall (1938) found that the osmotic resistance of the erythrocytes decreased and the mean corpuscular thickness increased during their passage through the spleen.

It is not quite certain, on the other hand, that the spleen is important for the normal destruction of erythrocytes, but there are observations which favour this view. According to Mann, Sheard, Bollman and Balder (1925) the bile pigment content of the blood increases during its passage through the spleen, but Rich (1925) observed no increase. Bergenhem and Fåhræus (1936) found that the corpuscles in the blood of the cat's spleen were more spherical, that is, thicker than other erythrocytes. According to Emerson, Shen and Castle (1946) and Young (1947) the thickness of normal human blood cells also increases in the spleen. Singer, Miller and Dameshek (1941) found a decreased hemolytic index after splenectomy and in general a marked increase in the number of »target cells» with increased osmotic resistance. According to

Singer (1945 a and b) this does not prove that the destruction of erythrocytes is decreased after splenectomy. Ask-Upmark (1935), on the other hand, observed that the red corpuscles in the blood of persons subjected to splenectomy had a normal osmotic resistance. The erythrocyte count was normal in general both in the former's and in Ask-Upmark's series. Singer and Weisz (1945) studied the life-span of erythrocytes in dogs before and after splenectomy and found no difference. Gordon and Kleinberg (1938) noticed that the erythrocytes lived longer in splenectomized animals (32 to 38 days) than in normal animals (22 to 28 days). Normal values were evidently not obtained by their methods — the figures for the life-span of erythrocytes were probably too low. They first induced an abnormal increase in the number of corpuscles by keeping the animal under low oxygen pressure and the organism always strives actively to counteract such a surplus of erythrocytes (Ponder 1948). We again find that the spleen takes an active part in the abnormally increased destruction of erythrocytes but whether it does so under normal conditions is doubtful.

HEMOLYSINS AND AGGLUTININS. THEIR ACTION ON RED BLOOD CORPUSCLES IN VIVO AND IN VITRO

There is no evidence, as already stated, of the role of hemolysins in the normal destruction of erythrocytes. Its mechanism has not been clarified so far; hence the numerous attempts to demonstrate the presence of hemolysins in the organism. They have been successful in that certain substances have been found whose effect in adequate concentration is hemolytic at least in vitro.

In certain cases the lecithin of the blood plasma inhibits hemolysis, e. g. the hemolytic action of bile salts, but some lecithins have hemolyzing qualities of their own (Ponder 1923 and 1948). When lecithin is affected by lecithinase, which is present, for instance, in snake poison, a very effective hemolyzing substance, lysolecithin, is formed. Its hemolytic action is evidently partly due to its capacity to dissolve cholesterol in the cell membranes and partly to the fact that it decreases the surface tension (Roy 1945). This substance was demonstrated by Berghem and Fåhræus (1936) in human blood and they observed that blood plasma, kept warm, forms lysolecithin if it is separated from the corpuscles, for instance through sedimentation. In other words, lysolecithin may form in all the blood depots of the body, especially in the spleen where plasma and corpuscles may be separated by sedimentation and also by other mechanisms (Knisely 1936, Björkman 1947). In familial hemolytic jaundice the resistance of the corpuscles to lysolecithin is reduced (Gripwall 1938, Singer 1940), and Gripwall's investigations show that the hemolyzing action of the spleen in this disease may be due to the formation of lysolecithin in this organ.

Maegraith, Findlay and Martin (1943) found that tissues of various organs in vitro form hemolysin when tissue slices, carefully washed in

physiological salt solution, are incubated at a temperature of 37°C. Ponder (1944) and Brückman and Wertheimer (1945) showed the same phenomenon. Ponder considers that the active hemolysin is lysolecithin or a closely related substance.

In vitro, the action of bile salts is strongly hemolytic, weaker in vivo (Ponder, Hyman and White 1941), and probably completely absent under normal conditions, as only very small amounts of these salts are present in the human blood (Ponder 1948). A hemolytic action in the circulating blood might sooner be attributed to the fatty acid salts which enter the blood with the chyle. The daily output of these salts would suffice to explain theoretically 8 to 35 per cent of the erythrocyte destruction (Freeman, Loewy and Johnson 1944). Their hemolytic action in the body is, however, inhibited in numerous ways, and, in consequence, their importance is still very doubtful.

We thus have very little knowledge of the activity of these potential hemolysins in the organism, but we know considerably more about corresponding phenomena in hemolytic disorders. In 1904, Donath and Landsteiner found an autohemolysin in the blood of patients with paroxysmal cold hemoglobinuria and proved that the hemolysis in these cases was due to the action of this hemolysin. Some years later Chaffard and his co-workers (1908 and 1909) found hemolysins of the amboceptor type in cases of acquired hemolytic jaundice. In 1940 Dameshek and Schwartz made close studies of the significance of hemolysins in this disease. In their comprehensive survey, they state that four or five different auto- or isohemolysins were found in conditions with pathologically increased erythrocyte destruction. In a large number of cases of acquired hemolytic jaundice and acute hemolytic anemia there were auto-antibodies in the form of agglutinins (Giordano and Blum 1937, Greenwald 1938, Dameshek and Schwartz 1940, Wasastjerna 1948 b, Du Pan 1948, Hahn and Lüttgens 1949, and others). In 11 cases of primary atypical pneumonia with a high cold agglutinin titer of the blood, Finland and his co-workers (1945) found signs of increased erythrocyte destruction.

In the last 12 years, an important group of hemolytic anemias, hemolytic disease of the newborn or erythroblastosis foetalis, has been explained (See surveys of Wiener 1946, Mollison, Mourant and Race 1948, Pickles 1949, Race and Sanger 1950, and others). This disease is considered due to immunization against the various Rh antigens. In this immunization, which is better understood than the origin of antibodies in any other type of hemolytic anemia, antibodies of different kinds occur, viz. »complete», »incomplete» or »blocking» and »third order antibodies», all of which have been demonstrated by different kinds of agglutination reactions. It is somewhat surprising, however, that very few reports in the comprehensive Rh literature deal with the remarkable fact that these antibodies which are demonstrable in vitro as agglutinins have the capacity

to dissolve red corpuscles in vivo. Also in many cases of acquired hemolytic jaundice, agglutinins, but no hemolysins are found. D a m e s h e k (1948) states that »agglutinins, when introduced into the circulation, result in hemolysis of red cells».

The mechanism by which these agglutinins act as hemolysins in vivo is not known, but some explanations have been attempted. For instance, S h e n, C a s t l e and F l e m i n g (1944) have shown that agglutinated erythrocytes have an increased mechanical fragility and are thus more easily affected by mechanical trauma in the circulation than free corpuscles. Large agglomerations of red cells have special difficulty in passing through the capillaries and are therefore easily influenced mechanically, as K n i s e l y and his co-workers (1947) found on microscopical examination of the circulating blood stream. B e r g e n h e m and F å h r a e u s (1936) and H a m and C a s t l e (1940 a and b) stated that incubated blood cells are not only influenced by lysolecithin in plasma, but also by a metabolic process whose action is similar to that of lysolecithin. The metabolic process also gradually brings about a change in the shape of the corpuscles, they become more spherical, and finally there is hemolysis. This also occurs if the corpuscles are completely liberated from plasma. H a m and C a s t l e's explanation of the action of agglutinins in vivo is that the intravascular agglutination affects the circulation and causes blood stasis in various organs, especially the spleen, which in its turn leads to hemolysis.

H i l l, H a b e r m a n and J o n e s (1948) have endeavoured to find Rh antibodies through hemolysis phenomena in vitro and state that they have been successful. Their »third order antibodies», for instance, dissolved red corpuscles in vitro. The hemolysis was, however, extremely slow and weak and demonstrable by special sensitive methods, but not until the elapse of 20 hours, hence there could have been no question of a direct hemolysis in an immunological meaning. The hemolysis was probably caused by the incubation, especially as a weak hemolysis occurred in the control tubes as well. If this interpretation is correct, the somewhat stronger hemolysis in tubes with corpuscles coated with antibodies would be due to these corpuscles being more easily influenced by incubation than ordinary corpuscles. According to H a m and C a s t l e (1940 b), erythrocytes taken from patients with familial hemolytic jaundice are more easily influenced than normal erythrocytes.

Many substances are known which have a more or less marked capacity to dissolve red corpuscles. Distilled water is probably the simplest of these substances. In it, or in hypotonic solutions the corpuscles swell because water passes into them and they become more and more spherical, finally the cell membranes burst and the cells are dissolved. Blood cells which are thicker than normal naturally become spherical sooner, hence they are hemolyzed more readily. They thus have a »reduced osmotic resistance». There is a definite relationship between the shape of the erythrocytes and the resistance. The more spherical the shape of the corpuscles the more easily are they hemolyzed in hypotonic solutions (H a d e n 1934,

H a m and C a s t l e 1940 b). However, the shape of the corpuscles is not changed only in distilled water and hypotonic solutions. A characteristic trait of hemolysis *in vitro* is that the corpuscles nearly always become more spherical before being dissolved, but their volume may increase simultaneously or no change may occur. In hypotonic solution the volume of the erythrocyte increases greatly, whereas, in solutions of hemolytic substances such as saponin, bile salts, fatty acid salts, lecithins, and amboceptor and complement, there is but a small increase or none at all. The reader is referred to P o n d e r's survey of 1948 for particulars regarding the hemolytic action of these substances and many other chemicals and drugs.

The most important among bacterial toxins which may give rise to hemolytic anemia are those formed by *Streptococcus hemolyticus* and *viridans* and *Clostridium welchii* (W i n t r o b e 1946). In septicæmia this last bacterium in particular may cause violent hemolysis (L e n h a r t z 1925).

The hemolysis caused by these or numerous other substances will not be dealt with in this work which is limited to certain examinations of the action of immune hemolysins *in vivo*. Some other hemolysins will be described only when a comparison between their actions and those of the immune hemolysins is of interest.

EXPERIMENTAL HEMOLYTIC ANEMIA

Among the various experiments with hemolysins *in vivo*, those made with immune hemolysin are firstly of hematological interest as parallels may be drawn between these and some forms of hemolytic anemia; secondly, the experiments may give some information regarding the destruction of normal erythrocytes which is still obscure; for that reason this account is mainly of the trials with immune hemolysin.

The work which has attracted the greatest interest in this field was published by D a m e s h e k and S c h w a r t z in 1938. They immunized rabbits by repeated injections of blood corpuscles of guinea-pigs, thus producing an immune serum able to agglutinate and, after the addition of complement, to dissolve guinea-pig corpuscles *in vitro*. The immune serum was injected intraperitoneally into guinea-pigs. Typical hemolytic anemia resulted, varying greatly in character according to the size and number of the doses. With average doses the blood picture showed pronounced spherocytosis during the first days, i. e. the diameter of the corpuscles was smaller than normal and their thickness increased; this was most distinctly observed on the third to fifth day. Parallel with this was a decrease in the resistance to hypotonic solutions. The number of corpuscles decreased during this period to about one million on the fifth to seventh day. Intense reticulocytosis occurred at about the same time and nucleated red cells appeared in the blood. The number of corpuscles then increased rapidly in the surviving animals. Measurement of erythrocytes during the regeneration period showed macrocytes in abundance because of the large diameter of the newly formed corpuscles — as large as that

of young corpuscles in untreated animals. During the period characterized by increased regeneration, while microcytes are still present in the blood, the erythrocyte measurement gave Price-Jones curves with two peaks. Large doses of immune serum caused a fulminant hemolytic anemia with hemoglobinemia and hemoglobinuria, and pronounced micro-spherocytosis; the osmotic resistance of the blood corpuscles was much reduced. In these cases there was no time for marked regeneration of erythrocytes before the animal died. On the other hand, treatment with numerous small doses gave rise to subacute hemolytic anemia in which the peripheral blood picture was often dominated by newly formed erythrocytes and therefore the picture was »pseudo-macrocytic«.

D a m e s h e k and S c h w a r t z were the first to study the blood picture and the characteristics of the corpuscles in experimental hemolytic anemia on a large scale. They showed that some conclusions may be made with regard to the mechanism in certain forms of hemolytic anemia in man. They point out first and foremost that a microspherocytic blood picture may be due to the corpuscles being influenced by a hemolysin. However, a long time before they published their results, several researchers had shown that the effect of anti-erythrocyte serum is hemolyzing also in vivo.

At the end of the last century B o r d e t discovered the antigenic qualities of the red blood cells. He repeatedly injected rabbit's blood into guinea-pigs. After the injections he observed specific hemolysin and agglutinin in the blood serum of the animals. The hemolysin was inactivated when heated and reactivated if fresh normal serum was added. As early as 1898, he made experiments with this immune serum in vivo and noted that it was highly poisonous when injected intravenously into rabbits. In the same year B e l f a n t i and C a r b o n e made similar experiments on animals of various species. They found that the toxic effect of the immune serum was specific to the species from which the blood had been taken.

C a n t a c u z e n e used the same kind of immune serum as B o r d e t and, in 1900, he compared the effect of doses of different sizes, injected intravenously or subcutaneously into rabbits. The animals died a few minutes after the injection of large doses. With a somewhat smaller dose the number of corpuscles decreased in a few days to one million or less, and intense regeneration set in. With very small, repeated doses he stimulated erythropoiesis and the number of corpuscles remained above normal.

K r a u s and S t e r n b e r g (1902) immunized rabbits by giving them injections of defibrinated dog's blood, thus obtaining an immune serum with which they treated dogs. If these dogs were given a large intravenous dose they died within 20 minutes, no hemolysis being observed. Somewhat smaller subcutaneous doses caused hemolytic anemia and jaundice. These authors also observed agglutination in blood samples taken from the animals treated, but they considered that the

corpuscles were not agglutinated in vivo — this probably occurred on the slide some time after the sample had been taken.

Levaditi (1902) injected hemolytic immune serum intraperitoneally into guinea-pigs which resulted in hemoglobinuria and anemia. He next studied preparations of the spleen under the microscope and noticed intense phagocytosis of red corpuscles. Dudgeon, Panton and Ross (1909) found the same kind of erythrophagocytosis in preparations of the spleen and lymphatic glands of guinea-pigs treated with immune hemolysin.

Joannovics (1904) made experiments with toluylendiamin and hemolytic immune serum of the same kind as Kraus and Sternberg used and induced hemolysis and jaundice in dogs with each of these substances separately. He had splenectomized some of the experimental animals beforehand and found that they were able to stand more toluylendiamin but less immune serum than those with an intact spleen. He only made a few parallel experiments, however, and no blood counts were done.

Muir and Mac Nee (1911) gave their rabbits intravenous injections of anti-erythrocyte serum obtained by immunizing a goat with washed blood corpuscles of rabbits. In the majority of cases hemoglobinemia and hemoglobinuria followed and the number of red blood corpuscles diminished during a period of about three days. These workers found that the hemolysis in vivo was not proportional to the doses given and calculated that a certain amount of immune serum hemolyzes more corpuscles in vivo than in vitro.

In 1913, Banti published an interesting work on the effect of hemolysins in vivo. He compared the effect of distilled water, anti-erythrocyte serum and toluylendiamin on dogs and rabbits. Intravenous injection of distilled water was immediately followed by hemoglobinuria and a slight, temporary decrease in the blood count. Subsequent to intravenous injection of immune serum hemolysis occurred in two stages, a rapid hemolysis following immediately after the injection and a slow hemolysis causing anemia which progressed over a period of several days. Both phases were followed by hemoglobinemia. In the corpuscles he found anisocytosis and poikilocytosis and reduced osmotic resistance. Like Muir and Mac Nee, Banti found a more intense hemolysis in vivo than might have been expected on the basis of the hemolysin titer in vitro. Some of the animals had been subjected to splenectomy and in these the hemolysis was weaker. The same difference between normal and splenectomized animals was also observed after injection of toluylendiamin, but the effect of distilled water did not differ whether the animals were splenectomized or not.

Filo's (1936) report was contradictory in that he found that splenectomy or »blocking» of the reticulo-endothelium by thorostrast did not protect guinea-pigs from the hemolytic action of immune hemolysins. He seems not to have made any close, quantitative comparison. He further observed a rapid fall in the erythrocyte count, microcytosis and leucocytosis after injecting immune serum

and, if the animals survived, there occurred in a few days a regeneration of red blood cells with intense reticulocytosis and normoblasts. If the animals died, an increased bilirubin content in the bile and erythrophagocytosis in the spleen were observed at the post mortem examination.

Since D a m e s h e k and S c h w a r t z published their work on experimental hemolytic anemia, several other authors have made similar experiments which largely verify their results. T i g e r t t, D u n c a n and H i g h t (1940) used dogs for their experiments and produced immune serum by immunizing rabbits with dog's blood corpuscles. Each dog was given an intraperitoneal injection of this serum. Within 12 hours there was a reduced osmotic resistance in the erythrocytes of the animals and a decreased erythrocyte diameter, the number of corpuscles decreasing somewhat later and reaching the minimum in three to six days. Hemoglobinemia and hemoglobinuria occurred in the majority of the animals.

N a p i e r and S e n G u p t a (1943) injected anti-erythrocyte serum intravenously into monkeys inducing hemolytic anemia. There followed a moderate decrease in the mean volume of the corpuscles. This is a phenomenon not observed by other workers in similar experiments. The red blood cells became more spherical and their osmotic resistance decreased simultaneously with the increase in the cell thickness. The spherocytes were gradually but not very rapidly destroyed as spherocytes were found in the experimental animal's blood as late as three weeks after the injection.

R i v a n o and M e n e g h i n i (1947), who made experiments on guinea-pigs, point out that the diameter of the erythrocytes decreased, and their resistance was reduced before their number was affected.

Also B a u m g a r t n e r (1947) used guinea-pigs for his experiments. He injected immune serum intraperitoneally in varying doses and induced, like D a m e s h e k and S c h w a r t z, hemolytic anemias of different types. B a u m g a r t n e r very closely examined the cells of the animal's spleen and liver, especially in smears. This procedure gives a more distinct picture of the separate cells than sections do. He observed pronounced phagocytosis of red corpuscles in the spleen and the liver. Endothelial cells, which had ingested solitary erythrocytes occurred, and in preparations of the spleen there were large macrophages filled with erythrocytes in different stages of destruction. Also in the peripheral blood there were erythrocytes which had been ingested by other cells, monocytes or neutrophil leucocytes.

B e s s i s and F r e i x a (1947) immunized rabbits with rat's blood corpuscles and injected the immune serum obtained in varying doses intraperitoneally into rats which resulted in hemolytic anemia accompanied by jaundice. In the spleen of the animals treated, intense phagocytosis of red blood cells was found and in some cases there was in the peripheral blood an abundance of monocytes which had ingested erythrocytes. They examined, in numerous cases, the fresh blood of the animals some hours after the injection and found agglutination of the corpuscles.

Microscopically they were malformed, often spindle-shaped and joined together in strands. When these corpuscles were seen against a dark background they were surrounded and united by a glue-like substance. These authors found that their immune serum was effective also when given by mouth, but only in newborn, not in adult rats.

Wasastjerna (1948 a) used guinea-pigs for his experiments and produced immune serum by Dameshek and Schwartz' method, but he gave most of the animals one intracardial dose. Only one hour later the mean corpuscular diameter was decreased in the majority, and in half the animals the erythrocyte count was reduced. After three hours the erythrocyte diameter was decreased and the erythrocyte count reduced in almost all the animals and after six hours in all of them. The fall in the erythrocyte count was, however, very moderate during the first six hours, averaging half a million. There was a further slow fall on the first day and a more rapid fall on the second day. The minimum erythrocyte count was generally reached in three to four days. The erythrocyte shape changed simultaneously, or somewhat more rapidly, approaching spherocytosis, i. e. blood cells with decreased diameter and increased thickness. Both Banti and Wasastjerna found that the action of the hemolysin was weaker in animals which had been splenectomized.

Tischendorf and Franke, like Bessis and Freixa, studied experimental hemolytic anemia in rats and their results verified those of the latter. They found that erythrocytes in the blood of the animals treated were destroyed partly through direct intravasal hemolysis, partly through phagocytosis. In their opinion, the spleen is not important in the origin of experimental hemolytic anemia as splenectomized rats acquired anemia of the same type as those with an intact spleen. Judging by Tischendorf's (1949) abstract, the erythrocyte destruction was not quantitatively compared in animals with a spleen and those that had had it removed. In these experiments, free agglutinin was found in the blood serum of the animals treated and if the erythrocyte destruction was great, there was free hemolysin.

Recently Young, Ervin and Yuile (1949) made some very interesting experiments on dogs. They did not produce immune serum by immunizing animals of another species but used the different blood groups present in dogs and immunized »Do-negative» dogs with »Do-positive» corpuscles. When »Do-positive» blood was injected into dogs, immunized in this manner, immediate, rapid hemolysis set in and all the corpuscles transferred were dissolved within 90 minutes, the majority within 10 minutes. The complement content in the recipients blood decreased simultaneously to about nil. But, when a »Do-positive» dog was given an injection of »anti-Do-serum», the recipient's corpuscles were slowly affected in a manner similar to that in the experiments by Dameshek and Schwartz' method, and the number of erythrocytes decreased gradually during a period of up to nine days. The complement content in the recipient's blood was barely

affected and no free antibodies were found in the animal's serum. Young, Ervin, Christian and Davies (1949) succeeded in immunizing a pregnant bitch by the method and signs of a hemolytic disease occurred in some of the puppies. In this case the antibodies were transferred to the fetal blood through the placenta in the same way as when hemolytic disease of the human newborn arises owing to Rh immunization of the mother.

There are numerous different kinds of drugs and chemicals with a hemolytic action in vivo and various experiments with these substances have been made on animals and human beings. The results obtained are not in general as interesting from a hematological point of view as those in trials with immune hemolysins. Some of the substances have a hemolyzing effect in vivo, but not in vitro (Isaak and Möckel 1911, Ponder 1948) which is interesting. This occurs, for instance with concanavalin A, a protein which Sumner and Howell (1936) isolated from jack beans (*Canavalia ensiformis*). In vitro it agglutinates the red corpuscles to a high degree, but in vivo it may give rise to hemolysis (Ham and Castle 1940 a and b, Dameshek and Miller 1943).

Soon after the discovery of bacterial hemolysins, their effect was studied in vivo and they were found to be hemolytic also in the circulating blood of experimental animals (Ch. Todd 1901, Kraus and Ludwig 1902). Hemolytic streptococci form two substances with hemolytic action which are termed streptolysin S and streptolysin O. Weld (1934 and 1935) found that the effect of the former was hemolyzing also in vivo. E. W. Todd (1938) studied the effect of these two hemolysins on injection into mice and found that they can both dissolve red blood corpuscles in the living organism. He considered the effect of streptolysin S purely hemolyzing while the action of streptolysin O was toxic in other respects also. Kalbak (1942), on the other hand, found no toxic effect of streptolysin O injected intravenously into rabbits and mice.

The hemolytic effect of saponin on blood corpuscles in vitro was closely studied by Ponder (Survey 1948). Together with Hyman and White (1941) he examined its action also in vivo and found that it was about 200 times weaker in vivo owing to three different factors, i. e. 1) a greater concentration of erythrocytes in vivo, 2) the inhibiting qualities of the serum, and 3) the saponin in vivo being partly absorbed by other cells than the red ones. As early as 1911, Isaak and Möckel found that saponin must be given intravenously in large doses in order to cause hemolysis in experimental animals although it is a very strong hemolysin in vitro.

Quite recently De Vries (1950) stated that he had been able to induce typical hemolytic anemia in rabbits by intravenous injection of lysolecithin; but splenectomy and hypercholesterolemia prevented its action.

CHAPTER II

THE PROBLEM

Several research-workers have induced experimental hemolytic anemia in animals — as already stated in the preceding chapter — by injecting anti-erythrocyte serum, and observations of great value for clinical hematology, especially the part termed immunohematology have been made. Yet many points in the pathogenesis of experimental hemolytic anemia are still obscure.

Firstly we must ask whether the hemolytic process in vivo and that in vitro are identical, i. e. whether exactly the same process takes place in the blood of the experimental animal after injection of hemolytic immune serum as in the tube in which red cells, antibodies and complement have been mixed. There is no proof that immune serum in vivo can influence directly, i. e. dissolve or agglutinate corpuscles in the same way as in vitro. In vivo the modifying effect of the living organism on hemolysis and hemagglutination has to be reckoned with. It may either promote or inhibit. As already mentioned, many authors have observed that the effect of immune hemolysin is stronger in vivo than in vitro whereas the hemolytic effect of saponin is weaker in vivo than in vitro. The purpose of this work is mainly to discover whether the living organism takes part in the hemolytic process in experimental hemolytic anemia, and if so, how? If this problem is solved, it will lead to better understanding of the mechanism of hemolysis in man, especially of hemolytic anemias with antibodies in the blood.

In order to distinguish the organism's effect on hemolysis in experimental hemolytic anemia we must first compare the effect of immune serum in vivo and in vitro, i. e. find an answer to the question: »Where does a given amount of immune serum dissolve more blood corpuscles, in vivo or in vitro?» It will not, however, suffice to assess the quantitative effect of immune serum in vitro with one titration method only, as some earlier investigators have done. Titrations must be done in various ways in order to evaluate how the different experimental conditions influence the results in vitro, and a direct comparison of the effect in vivo and that on whole blood in vitro must be made. I have previously (Wasastjerna 1948 a) made some experiments of this kind which are now checked and enlarged. Formerly I used heparin blood in vitro, but as it is not suitable because it inhibits hemolysis (Bergenhelm 1938) and the complement effect of normal serum to some

extent (Wising 1937, Hedenius and Snellman 1937), the whole blood tests will now be made with defibrinated blood. My values for the blood volume of guinea-pigs were probably also too high and an improved technique will therefore now be used for these determinations.

If the investigations show that the living organism either promotes or inhibits hemolysis in vivo — the manner in which it takes place must be discovered. Since the spleen is considered important for hemolysis, some parallel tests with normal and splenectomized guinea-pigs will be made in order to find a reply to the question: »Does a given amount of immune serum dissolve as many corpuscles in splenectomized animals as in those with an intact spleen?» Some investigations of this kind have been made before, but the materials were small and the question requires further elucidation, particularly as the results are contradictory.

In the animal experiments with immune serum, not only the erythrocyte count will be followed in order to form an idea of the quantitative effect of immune serum in vitro, but the shape of the corpuscles will also be observed. As already stated, spherocytosis arises in experimental hemolytic anemia and may be interesting for various reasons. It may be the result of a small amount of hemolysin being present, the action of which is too weak to dissolve corpuscles (Ponder 1948, Estren and Dameshek 1949), or it may be thought that it has a pathogenetic significance in the origin of anemia; this is probably the case in familial hemolytic jaundice in man. As the corpuscular fragility in hypotonic solutions is proportional to the spherocytosis (Haden 1934, Castle and Daland 1937), an idea of the fragility, which will not be determined now, will be obtained.

Anti-erythrocyte sera have not only a hemolyzing quality, but also a hemagglutinating capacity, and we may ask whether it is not the agglutinins which cause hemolysis in vivo. Let us observe whether agglutination occurs in the animal's blood after injection of serum and compare the hemagglutinating capacity of immune sera in vivo and in vitro. The question of the effect of agglutinins in vivo is of great interest as agglutinins are found more often than hemolysins in hemolytic anemias in man.

Some authors consider that opsonins — and not hemolysins or agglutinins — are of the greatest pathogenetic importance in experimental hemolytic anemia. Whether there are actually three different substances or only different effects due to one and the same substance will not be discussed here, but the effect of the immune opsonins will be observed for phagocytosis of red blood corpuscles in blood smears and spleen preparations of the animals treated.

Finally, the effect of immune serum on red corpuscles will be compared with that of pure hemolysins, i. e. saponin and streptolysin, and with an agglutinin, i. e. extract of beans which does not hemolyze in vitro. These comparisons will be made in vivo and in vitro, and for that reason experiments on animals, similar to those with immune serum, will be made with these substances

CHAPTER III

THE METHOD

EXPERIMENTAL ANIMALS

Adult guinea-pigs of about 500 gm., nearly all between 400 and 600 gm. in weight were used.

Some were splenectomized. The operation was done under combined urethane-ether-anesthesia, 0.4 to 0.5 ml. of 25 per cent urethane solution per 100 gm. body-weight first being given intraperitoneally and then ether as much as required. The abdomen was opened by medial incision and catgut used for suturing. The technique of the operation is simple, but caution and absolute sterility is required. The splenectomized animals were generally used about one month, at the earliest 16 days after the operation (one case).

IMMUNE SERUM

Immune serum with antibodies to guinea-pig corpuscles was produced according to D a m e s h e k and S c h w a r t z (1938). Rabbits were repeatedly given intravenous injections of washed guinea-pig corpuscles. When the titer of the rabbit serum was sufficiently high the blood was drawn, the serum separated, and inactivated for 30 minutes at 56°C. and packed in 1—2 ml. glass ampulles, all this under aseptic conditions. No preservation substance was added. The sterility of the serum was checked and the ampulles kept in a refrigerator.

The hemolysin content of the immune sera obtained was determined by titration according to the method in D a m e s h e k and S c h w a r t z's work. The serum dilutions were 1:2:4:8:16, etc. To each tube containing 0.5 ml. of serum dilution was added as complement 0.5 ml. of fresh guinea-pig serum diluted 1:10, 1.5 ml. of physiological salt solution and 0.5 ml. of a 2 per cent suspension of washed guinea-pig corpuscles in saline. When the other ingredients were added the serum was thus diluted a further six times. The tubes were shaken and allowed to stand for two hours at 37°C. in a thermostat and then the titer was read. At each titration a record was made of the *final* serum dilution in the last tube in which all the corpuscles were dissolved, and in the last tube in which hemolysis was visible. The former dilution was termed »total hemolysis titer» and the latter »visible hemolysis

titer». The first titer was generally easily read, but as it was sometimes difficult to determine which tube was the last one in which hemolysis was visible, the hemolysin content in this work will generally be given as a total hemolysis titer if no special titer is referred to. For hemolysin doses the term hemolytic units (H. U.) will be used for the sake of simplicity; in this work this is the amount of hemolysin which can dissolve 0.1 ml. of a 2 per cent suspension of guinea-pig corpuscles according to the above titration scheme. This amount is thus contained in 0.1 ml. of the greatest dilution of serum which, after the addition of complement, saline, and corpuscle suspension gives total hemolysis. For instance, if the titer of a serum is 1:48, 0.1 ml. of serum contains $48/6 = 8$ H. U.

The agglutinin titer was determined by the same method as hemolysin. Parallel tests were first made with physiological salt solution instead of complement, but as the results were always the same as in titration with complement present, the parallel tests were later abandoned and the agglutinin titer read at the same time as the hemolysin titer in the same series of tubes. The agglutinin titer was always higher than the hemolysin titer. Before reading the agglutinin titer the tubes were so vigorously shaken that all the corpuscles were suspended and the titer given was that of the final dilution in the last tube in which the suspension was macroscopically distinctly granular. The agglutination was always checked microscopically and at least half of the corpuscles required to be united in large or small agglomerations. The titer limit read in this way was quite distinct. In the following, or in the two following tubes, small solitary agglomerations were then generally visible microscopically, while the majority of the corpuscles were free.

All the sera used were repeatedly titrated during the investigation. No sinking tendency was observed, neither in the hemolysin nor the agglutinin titer. They did not all give exactly the same results, however, a variation was sometimes observed, but never more than 1:2, which is the normal error in titrations of this type (Boyd 1947). In these cases the value most often obtained is given as the titer.

SAPONIN, STREPTOLYSIN, AND EXTRACT OF BEANS

Besides the experiments with immune serum, trials were made with two other hemolysins, i. e. saponin and streptococcal hemolysin. The former was »Saponinum purissimum» (Baker) dissolved in physiological salt solution. The hemolytic activity of these solutions was found to be unchanged for at least one month but solutions older than a fortnight were not used. They were sterilized by heating to 90°C. for 5 minutes; this did not much affect their hemolyzing capacity.

The streptococcal hemolysin consisted of reduced streptolysin O which was produced by Kalk's (1942) method at the Sero-Bacteriological Institute of the University of Helsingfors and kindly placed at my disposal by Dr. Nils Oker-Blom.

Other experiments were made with pure agglutinin. An extract of beans was used for this purpose: beans (*Phaseolus vulgaris*) were crushed in a mortar to fine powder, 10 gm. of which was mixed with 90 gm. of physiological salt solution and allowed to stand for two hours in a thermostat at 37°C. and overnight in a refrigerator. The suspension was then centrifuged. The slightly opalescent fluid was passed through a Seitz filter, checked for sterility and placed in sterile 5 ml. injection flasks. It was used within a week after production; its titer remained unchanged during this time.

All these substances were titrated by the same method as the immune sera, but saline was used instead of complement, and the titer notations were made according to the same principles.

INJECTION TECHNIQUE

In order to compare the effect of hemolysins *in vivo* and *in vitro* the injections must be given direct into the blood, either intravenously or intracardially. With guinea-pigs the latter technique is easier, yet the intravenous route was selected in this investigation in order to make certain that the whole amount of fluid entered the blood directly. The injection was given in vena jugularis which was laid bare by a longitudinal incision on the throat.

However, this procedure requires anesthesia. The serum was quickly injected and well tolerated, hence a mild ether anesthesia sufficed. But all the other substances caused severe shock if injected rapidly, and as there is difficulty in keeping a guinea-pig under ether anesthesia for a long period, urethane and ether were given in these cases, i. e. 0.35 ml. of a 25 per cent urethane solution per 100 gm. body-weight intraperitoneally and ether as required. All injections of saponin, streptolysin or bean extract were then given slowly — during 20 to 30 minutes. The intraperitoneal injections were of course given without anesthesia. As the anesthesia may influence the blood picture to some extent, some control tests were made with intravenous injections of saline under the same kind of anesthesia as described above. Particulars regarding these tests are given in Chapter IV.

DETERMINATION OF THE BLOOD VOLUME

The blood volume was also determined under anesthesia of the same kind, and assessed by determining the dilution of Evan's blue in the blood after an intravenous injection of the dye (Levinson and MacFate 1946). Naturally the method was somewhat modified for guinea-pigs. The heart was punctured and 3 ml. of blood aspirated. An intravenous injection of 3 ml. of a sterile dilution of Evan's blue in saline was given. The concentration of the dilution was 1:2 000 and the injection was given during five minutes. The heart was again punctured five minutes after the injection and 3 ml. of blood aspirated. The hematocrit value was determined in

one drop of blood from this sample. The two blood samples were then allowed to coagulate and the colour of the serum from the second sample was determined colorimetrically with the aid of a Lumetron photocell photometer with an orange filter (620) and an absorption cell of 1 mm.'s depth, serum from the first blood sample being used as reference liquid. The dilution of the dye was read on a calibration curve previously prepared on semi-logarithmic millimetre paper for known dilutions of the dye in guinea-pig serum. The curve was based on the means of two different series of determinations which agreed well, and the known dilutions used corresponded to the dilution 1:2 000, diluted 4, 6, 8, 10, 12 and 14 times. After having calculated the plasma volume in this manner and determined the hematocrit value (Vol. %), the blood volume was computed according to the expression

$$\frac{100 \times \text{plasma volume}}{100 - \text{Vol. \%}}$$

Unfortunately more than one blood sample could not be taken after the injection as the results would have been affected too much by the repeated drawing of 3 ml. of blood. Therefore no time interpolation could be done. The quantity of blood in the samples was kept at 3 ml. in order to obtain 1 ml. of serum for the absorption cell which could take this amount, and to avoid dilution of the serum and consequential possibilities of error. The method described may not be quite satisfactory, but its exactness, which has not been closely examined, will no doubt suffice for this work as only an approximate idea of the blood volume in guinea-pigs was required to compare the tests *in vivo* and *in vitro*.

BLOOD EXAMINATIONS

In most of the experiments, erythrocyte counts (R. B. C.) were done and the hematocrit value (Vol. %), erythrocyte diameter, and the percentage of reticulocytes (Retic.) determined before the treatment and at different intervals after the treatment. All the samples were taken through a small, superficial incision through one of the small veins of the ear.

Bürker's counting chamber was used for the erythrocyte counts; the blood was diluted with Hayem's solution in the ratio 1:200. Both sides were filled, the erythrocytes enumerated in both fields in 0.02 cu.mm. fluid, and the mean calculated. All the samples of the same animal were taken with the same pipette and all counts were done with the same counting chamber by the same person.

The volume of packed red cells was determined with van Allen's hematocrit tube, Warburg's solution, i. e. an isoton buffered oxalate solution (Meulengracht and Gram 1930) was used for dilution, and the tubes centrifuged for 30 minutes with a rapidity of about 3 000 turns per minute. Two tubes were filled each time and the mean of the readings recorded. The erythrocyte diameter was measured with a controlled ocular micrometer in stained smears. Two hundred

red corpuscles were measured, the same microscope and ocular micrometer, whose correction figure was 1.0, were always used. Erythrocytes with a diameter of $5\ \mu$ or less were termed microcytes (Micr.).

The mean corpuscular volume (M. C. V.) was calculated according to the expression

$$\text{M.C.V.} = \frac{\text{Vol. \%}}{100 \times \text{R.B.C.}} \times 10^9 \text{ cu. } \mu$$

and the mean thickness (M. C. T.) according to the expression

$$\text{M.C.T.} = \frac{\text{M.C.V.}}{\pi \times \left(\frac{\text{M.C.D.}}{2} \right)^2}$$

in which M. C. D. is the mean diameter of the corpuscles. The relation of the mean thickness to the mean diameter of the corpuscles is termed spherical index (Sph. I.), in accordance with Heilmeyer (1936-37).

The reticulocytes were vitally stained with brilliant cresyl blue, smears were made and after-stained with Giemsa solution. Reticulocyte counts per 1000 erythrocytes were done.

Blood samples were also taken for examination with respect to hemagglutination (Aggl.). This was done by the simple process of mixing 20 cu. mm. of blood and 10 cu. mm. of 3.8 per cent sodium citrate solution on a slide; the drop was allowed to stand a few minutes at room temperature. It was shaken a few times and then studied macro- and microscopically.

URINE EXAMINATIONS

Hemoglobinuria (Hgb. uria) was determined macroscopically and benzidine tests were made in doubtful cases. Microscopical controls were generally made to make sure that hematuria did not occur.

Urobilin in the urine was shown qualitatively by Schlesinger's test (Schl.): One drop of spirit of iodine and 2 ml. of saturated spirit solution of zinc acetate were added to 2 ml. of urine. The mixture was allowed to stand for a while. If the clear fluid showed green fluorescence after the filtration, the test was considered positive. It was generally negative in urine of untreated animals, in exceptional cases slightly positive, hence a moderate or highly positive test was considered a result of the treatment.

CHAPTER IV

THE NUMBER, VOLUME AND DIMENSIONS OF THE RED BLOOD CORPUSCLES IN GUINEA-PIGS, AND THE VARIATIONS IN THEIR BLOOD VALUES

THE ERYTHROCYTES IN UNTREATED AND IN SPLENECTOMIZED GUINEA-PIGS

The material contains 52 healthy guinea-pigs which were not splenectomized. Blood samples were drawn for enumeration of erythrocytes and determination of their volume and dimensions. The pretreatment values are given as initial values in Tables 2, 6—7 and 9—12. The means of the blood values and their standard deviations from the mean of the blood values of the 52 untreated animals and of 16 guinea-pigs, splenectomized 16 to 66 days before taking the samples, are given in Table 1. The mean errors of the means were calculated to allow of comparison of the two groups. These, and the mean errors for the differences between the average blood values in the two groups are also given in Table 1. The differences between the means of the number of erythrocytes, their mean volume, mean diameter and spherical index are equal to, or less than the respective

TABLE 1.
NORMAL VALUES FOR UNTREATED AND FOR SPLENECTOMIZED GUINEA-PIGS

	R.B.C.	M.C.V.	M.C.D.	M.C.T.	Sph. I.
Mean values for 52 normal guinea-pigs (M_1)	5.41	83.4 cu. μ	7.17 μ	2.07 μ	0.29
Standard deviation from the mean ...	± 0.53	± 5.3 »	± 0.20 »	± 0.14 »	± 0.07
Mean error of the mean (σM_1)	± 0.07	± 0.73 »	± 0.03 »	± 0.02 »	± 0.01
Mean values for 16 splenectomized guinea-pigs (M_2)	5.36	83.2 »	7.22 »	2.01 »	0.28
Standard deviation from the mean ...	± 0.46	± 6.7 »	± 0.22 »	± 0.14 »	± 0.02
Mean error of the mean (σM_2)	± 0.12	± 1.73 »	± 0.06 »	± 0.04 »	± 0.01
$M_1 - M_2$	0.05	0.02 »	-0.05 »	0.06 »	0.01
$\sigma (M_1 - M_2)$	± 0.14	± 1.86 »	± 0.06 »	± 0.04 »	± 0.01

mean errors. Only the difference between the average mean thickness of the erythrocytes in the two groups may perhaps be of interest, but as it is as small as 0.06μ and the mean error is 0.04μ , there is only an 87 per cent probability of there being a difference. Judging from this very small material it is thus possible — but not certain — that the mean corpuscular thickness is less in animals without a spleen than in those with the organ intact. Whether this is true or not, the difference is insignificant.

Although the mean corpuscular diameter is about equal in normal and splenectomized animals in this material, there seems to be a difference in the number of microcytes, i. e. corpuscles with the diameter 5μ or less. The measurements showed that most of the non-splenectomized animals lacked these erythrocytes. Twenty of the 52 animals had 0.5 or 1 per cent microcytes in the smears and none had more than 1 per cent. On the other hand, five of the 16 splenectomized animals had over 1 per cent microcytes in the blood; thus not a constant finding in the splenectomized animals but, as none of the 52 with an intact spleen had more than 1 per cent microcytes, the larger number of these cells in the five cases should be considered due to the splenectomy.

The number of reticulocytes varied greatly in the different animals — from 0 to 5 per cent — exceeding 3 per cent only in three cases. An increase in reticulocytes to over 3 per cent after treatment was considered »reticulocytosis» in these experiments, providing the value was at least twice the initial value.

CONTROL EXPERIMENTS

For the purpose of discovering the influence of a hemolysin on the blood count in an animal, one or more blood samples have to be drawn before the treatment in order to obtain an initial value. Hemolysin is injected and the red blood cells enumerated in samples taken at given intervals after the injection. All the changes in the erythrocyte count observed cannot, of course, be considered a specific effect of the hemolysin — they may also be caused by other factors, the most important of which are methodical errors in taking the sample and enumeration of the corpuscles, physiological variations in the animal, non-specific effects of the amount of fluid injected, and the injection technique, i. e. in this work the anesthesia, the injury which may be caused in laying bare the vein in intravenous injection, and the trauma in intraperitoneal injection. Not only the number of erythrocytes, but all other blood values may be affected by these factors.

Control tests on 10 animals were made in order to gain some knowledge of how all these factors together affect the different blood values. Eight guinea-pigs were given intravenous injections of saline, the vein was laid bare, and anesthesia given as in the actual experiments. Four of these animals were given urethane and ether and four only ether. Two were repeatedly given intraperitoneal injections of physiological salt solution. The doses varied and were selected to correspond

with the amount of fluid given in some of the actual experiments. Blood samples were taken before and after the treatment at the same intervals as after the hemolysin injections. The blood values obtained, seen in Table 2, showed some moderate variations. No functional connection was observed between the time elapsed after the treatment and the variations in the blood values, hence no attention was paid to the time in the statistical assessment of the results — all post-treatment values were considered of equal importance and related only to the initial value.

A total of 56 values were obtained for the erythrocyte number and 54 for the mean corpuscular volume, mean diameter, mean thickness and spherical index after the treatment. The variations in all these values in relation to the respective initial values, and the arithmetic means of the variations and the standard deviation from the initial values were calculated. The results of these computations and the mean errors of the means of the variations are given in Table 3. The standard deviations, multiplied by 2.58, which corresponds to the confidence coefficient 0.99, are also included in the table.

The first column in Table 3 shows that the 56 erythrocyte values after treatment were on an average 0.43 per cent lower than the initial values but as the mean error in this material is 1.94 per cent, the decrease is not significant. It is thus observed that the injections of saline and the other part of the treatment caused no significant change in the erythrocyte count. Nor are the changes in the mean corpuscular volume, mean thickness or spherical index statistically significant. But importance should be attached to the changes in the mean corpuscular diameter which was 0.57 per cent larger than before the treatment in an average of 54 observations. The difference is not great, but, as the mean error is only 0.22 per cent, there is a 99 per cent probability that the post-treatment diameter was larger than the pre-treatment diameter. There was thus no significant change — except in the erythrocyte diameter — in the blood values after the treatment in relation to the initial values. The change in the mean diameter was very small, yet statistically significant. In the tests with hemolysin injections the changes were much greater and in the opposite direction.

The first line in Table 3 gives the standard deviations from the initial values under the conditions occurring in these tests, and the second line the same figures multiplied by 2.58. The latter values correspond, as already mentioned, to the confidence coefficient 0.99. This denotes that the probability of an observation value, under the conditions mentioned, differing from the initial value by these percentages or more, is only 1:100. When the significance of one single observation has to be assessed in the actual experiments with hemolysin injections there is a 99 per cent certainty that a deviation from the initial value is specific and due to the hemolysin if it is more than 2.58 times the standard deviation in the control tests. In this investigation the deviation from the initial value in any one case must be more than 2.58 times greater than the standard deviation in the control tests to be considered significant.

TABLE 2.
CONTROLS, VARIATIONS IN BLOOD VALUES AFTER INJECTION OF
PHYSIOLOGICAL SALT SOLUTION

No.	Anesthesia	Saline ml./100 gm.		Before treat- ment	After (first) injection							
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d
142	Urethane + ether	0.1 ml.	R.B.C.	5.08	4.92	5.36	5.22	4.97		5.19		5.15
			M.C.V.	85	87	79	83	86		81		84
			M.C.D.	6.97	6.94	7.10	7.06	7.11		6.95		6.98
			Micr. %	0.5	0.5	0	0	0		0		0
			M.C.T.	2.2	2.3	2.0	2.1	2.2		2.1		2.2
			Sph. I.	0.32	0.33	0.28	0.30	0.31		0.30		0.32
143	»	0.5 ml.	R.B.C.	4.81	4.69	5.16	4.78	5.00		4.63		4.99
			M.C.V.	94	89	86	87	85		91		85
			M.C.D.	7.00	7.02	7.12	7.00	7.12		7.13		7.02
			Micr. %	0.5	0	0	0	0		0		0
			M.C.T.	2.4	2.3	2.2	2.3	2.1		2.3		2.2
			Sph. I.	0.34	0.33	0.31	0.33	0.30		0.32		0.31
148	»	1.0 ml.	R.B.C.	5.54	5.86	5.58	5.68	5.48		5.13		5.76
			M.C.V.	81	80	82	82	86		82		82
			M.C.D.	6.74	6.72	6.83	6.97	6.85		6.70		6.71
			Micr. %	1	1.5	0.5	0	0.5		0.5		1
			M.C.T.	2.3	2.3	2.2	2.2	2.3		2.3		2.3
			Sph. I.	0.34	0.34	0.32	0.32	0.34		0.34		0.34
149	»	0.3 ml.	R.B.C.	5.72	5.52	5.35	5.50	5.05		5.49		5.32
			M.C.V.	80	80	84	84	84		78		79
			M.C.D.	6.79	6.87	6.84	6.78	6.79		6.67		6.78
			Micr. %	1	0.5	0.5	0	0.5		0.5		1
			M.C.T.	2.2	2.2	2.3	2.3	2.3		2.2		2.2
			Sph. I.	0.32	0.32	0.34	0.34	0.34		0.33		0.32
146	Ether	0.2 ml.	R.B.C.	4.82	4.85	4.64	4.71	4.91		4.99		4.90
			M.C.V.	94	88	96	95	93		91		94
			M.C.D.	7.14	7.18	7.20	7.23	7.22		7.27		7.32
			Micr. %	0.5	0.5	0	0	0		0.5		0
			M.C.T.	2.3	2.2	2.4	2.3	2.3		2.2		2.2
			Sph. I.	0.32	0.31	0.33	0.32	0.32		0.30		0.30
147	»	0.1 ml.	R.B.C.	4.61	4.63	4.49	4.70	4.93		4.68		4.88
			M.C.V.	85	86	87	90	91		95		92
			M.C.D.	7.14	7.17	7.16	7.25	7.31		7.33		7.26
			Micr. %	0.5	0	0	0	0		0		0
			M.C.T.	2.1	2.1	2.2	2.2	2.2		2.2		2.2
			Sph. I.	0.29	0.29	0.31	0.30	0.30		0.30		0.30

TABLE 2 (continued)

No.	Anesthesia	Saline ml./100 gm.		Before treat- ment	After (first) injection								
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d
151	Ether	0.15 ml.	R.B.C.	5.72	5.42	5.61	5.59	5.70		5.64		5.51	
			M.C.V.	81	90	81	86	86		80		83	
			M.C.D.	6.95	6.97	6.98	6.93	6.96		6.84		6.89	
			Micr. %	0	0	0.5	0	0		0.5		0	
			M.C.T.	2.1	2.4	2.1	2.3	2.3		2.2		2.2	
			Sph. I.	0.30	0.34	0.30	0.33	0.33		0.32		0.32	
153	»	0.5 ml.	R.B.C.	5.90	5.66	5.81	5.75	5.88		5.36		5.45	
			M.C.V.	80	80	82	81	80		83		78	
			M.C.D.	6.91	6.72	6.95	6.95	6.81		6.64		6.75	
			Micr. %	0.5	0	0	0	0.5		1.5		0.5	
			M.C.T.	2.1	2.3	2.2	2.1	2.2		2.4		2.2	
			Sph. I.	0.30	0.34	0.32	0.30	0.32		0.36		0.33	
144	—	0.5 ml. × 6 intraperit.	R.B.C.	5.16			5.60		5.14		5.21		4.87
			M.C.V.	83			87		92		87		
			M.C.D.	7.11			7.14		7.18		7.28		
			Micr. %	0			0.5		0		0.5		
			M.C.T.	2.1			2.2		2.3		2.1		
			Sph. I.	0.30			0.31		0.32		0.29		
145	—	»	R.B.C.	4.87			5.10		4.67		5.20		5.07
			M.C.V.	86			87		87		85		
			M.C.D.	6.97			7.01		7.12		7.15		
			Micr. %	0			0.5		0		0		
			M.C.T.	2.3			2.2		2.2		2.1		
			Sph. I.	0.33			0.31		0.31		0.29		

h = hours

d = days

R.B.C. = mill. red blood corpuscles per cu.mm.

Micr. = microcytes

M.C.V. = mean corpuscular volume in cu. μ M.C.D. = mean corpuscular diameter in μ M.C.T. = mean corpuscular thickness in μ

Sph. I. = spherical index

TABLE 3,
VARIATIONS IN CONTROL EXPERIMENTS

	R.B.C.	M.C.V.	M.C.D.	M.C.T.	Sph. I.
Standard deviation in % of initial value	± 4.5	± 5.0	± 1.6	± 5.9	± 6.9
2.58 × standard deviation (confidence coeff. 0.99)	± 11.6	± 12.9	± 4.1	± 15.2	± 17.8
Mean change in % of initial value ...	— 0.43	+ 0.87	+ 0.57	+ 0.94	+ 0.69
Mean error of mean change	± 1.94	± 0.67	± 0.22	± 0.81	± 0.95

SUMMARY OF CHAPTER IV

In untreated normal guinea-pigs and in splenectomized guinea-pigs the erythrocyte count, mean corpuscular volume, mean diameter, mean thickness and spherical index were about equal. Some of the splenectomized animals showed an increased number of microcytes in comparison with those who had not been splenectomized.

Control tests were made on 10 guinea-pigs. Physiological salt solution was injected under the same conditions as in the experiments with hemolysin injections. The standard deviations from the initial value in the control tests were calculated. In this work a deviation in any one case is considered significant only if it is more than 2.58 times greater than the standard deviation in the control tests.

CHAPTER V

ANIMAL EXPERIMENTS WITH IMMUNE SERUM

THE GENERAL PICTURE AND THE COURSE OF EXPERIMENTAL HEMOLYTIC ANEMIA

Five immune sera of different titers were obtained by immunizing five rabbits with guinea-pig corpuscles. Three of these sera were used for experiments in vivo and in vitro. As there is no proof of their effect in vivo being directly proportional to their titer in vitro, the various sera, whose titers are given in Table 4, were used in different experimental series.

TABLE 4.
TITERS OF ANTI-ERYTHROCYTE SERA

	Total hemolysis titer	Visible hemolysis titer	H.U. per 0.1 ml. serum	Agglutination titer
Serum II	1: 48	1:1 536	8	1:3 072
» IV	1: 24	1: 384	4	1:1 536
» V	1:192	1:3 072	32	1:6 144

Thirty-two guinea-pigs were given an intravenous injection of immune serum. Most of the animals were given doses which induced severe hemolytic anemia but did not kill them. An example will best illustrate an experimental hemolytic anemia of this kind. Table 5 shows a typical experiment record and Figs. 1 to 5 the shape and diameter of the erythrocytes at different intervals after the injection in this experiment. All the 32 experiments are given in Table 6, the doses, changes in erythrocyte count, and corpuscular dimensions after the injection being included.

Hemoglobin was found in the urine of most guinea-pigs as soon as two or three hours after the injection. The hemoglobinuria lasted one to three days. During the first or the second day the urobilin in the urine was increased, and Schlesinger's urobilin reaction was positive for three to five days. Small doses of immune serum caused urobilinuria, but not hemoglobinuria, providing the doses were large enough to induce distinct anemia.

TABLE 5.

RECORD OF EXPERIMENT No. 85. INTRAVENOUS INJECTION OF 6 H.U.
PER 100 GM. OF SERUM II

Time	R.B.C.	Vol. %	Erythrocyte diameter in μ								M.C.V.	M.C.T.	Sph. I.	Retic.	Hgb. uria	Schl.	Aggl.
			5	6	7	8	9	10	11	M.C.D.							
Before inj.	5.29	44.5		10	57.5	30	2.5			7.25	84	2.0	0.28	1.3 %	—	—	—
3 h after inj.	5.10	40	2.5	27	54	15	1.5			6.86	78	2.1	0.31		+		+
1 d » »	3.81	29.5	12	36	39.5	12	0.5			6.53	77	2.3	0.35		+	+	+
2 d » »	2.40	18.5	28.5	47.5	17.5	6	0.5			6.03	77	2.7	0.45		+	++	+
3 d » »	1.82	17	14	43	22.5	9	8.5	3		6.64	94	2.7	0.41	10.5 %	—	+	(+)
4 d » »	1.70																
5 d » »	2.17	23	7.5	43	17	13	11.5	7	1	7.03	106	2.7	0.38	15 %			—
9 d » »	4.46																

Vol. % = hematocrit value

Hgb. uria = hemoglobinuria

Schl. = urobilinuria, shown by Schlesinger's test

Aggl. = agglutination of blood corpuscles

Retic. = reticulocyte value in %

The first blood samples were generally taken three hours after the injection in order to record the immediate (direct) effect of hemolysin in the blood. This blood count often showed a very slight decrease in the number of erythrocytes, some hundred thousand per cu. mm., but in some cases there was no decrease and in some there was even an increase, probably due to the serum injected and the surgical interference causing a slight shock and the amount of circulating blood plasma in consequence being reduced. Daily blood counts were done for five days, as a rule on the seventh day after the injection and sometimes once or twice at a later date. The fall in the erythrocyte count was fairly small during the first three hours, but after one day the number had decreased considerably. The lowest number of red blood cells was counted three to five days, generally four days after the injection. Then followed a rapid increase in the surviving animals. Six guinea-pigs died and as their erythrocyte count was very low before death, anemia was assumed to be the cause of death. Twenty-six animals survived, all of them showing increasing erythrocyte counts at the end of the observation time, i. e. five to ten days after the injection. Calculating the daily decrease in the number of corpuscles in these 26 cases it will be found that the decrease was greatest during the second day in 13 cases, during the first day in 10 cases, and during the third day in three cases.

A moderate change in the shape of the cells was observed as a rule three hours after the injection. In many cases there were then a few per cent microcytes in the blood and the spherical index of the erythrocytes was somewhat increased owing to the mean diameter being decreased and the thickness increased. The changes

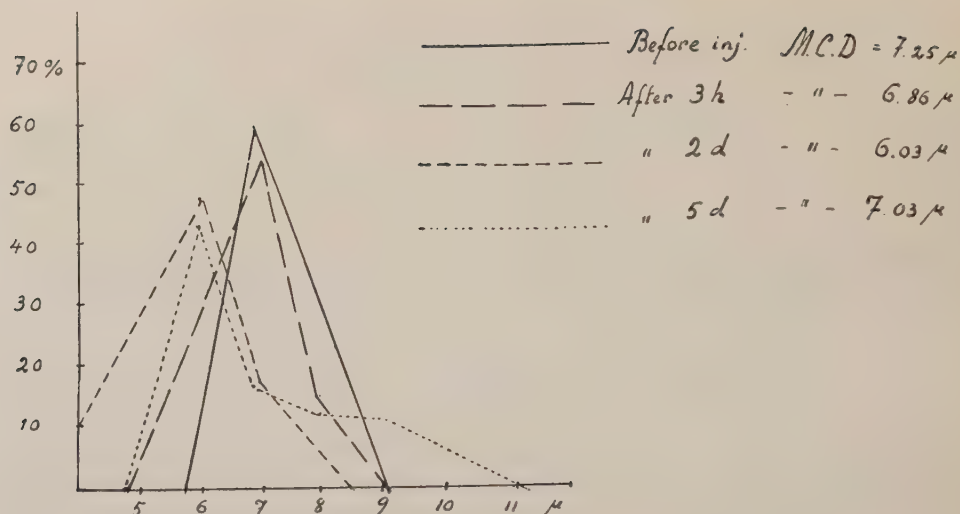


Fig. 1.

Distribution of erythrocyte diameter in per cent before and after intravenous injection of anti-erythrocyte serum (Price-Jones curves). Experiment No. 85.

in the shape continued, the diameter decreasing and the thickness increasing so that the highest spherical index was generally observed two days after the injection. At that time also the number of microcytes was highest as a rule. In several animals the microcytes increased from 0 to 1 per cent before the injection, to values ranging between 20 and 30 per cent. The largest number of microcytes observed was 64 per cent. As Heilmeyer (1936—37) pointed out, the best idea of the spherocytosis is obtained by calculating the spherical index of the corpuscles. It frequently increased very much, sometimes to twice the initial value. In many cases the erythrocytes did not attain their largest mean thickness until after five days, owing to a large number of newly formed cells with large diameters and great thickness then being present.

During the first days after the injection there was often some variation in the mean corpuscular volume. It generally decreased somewhat, but this was not a constant phenomenon in all the experiments. On the other hand, an appreciable increase in the mean volume was observed when the regeneration of blood cells started. This regeneration was also evidenced as a marked reticulocytosis. In all the experiments in which fairly severe anemia arose, a great increase in the number of reticulocytes, up to 20 per cent, was observed five days after the injection and sometimes sooner. The diameters which in the first measurements after the injection were decreased in all the animals varied greatly in the beginning of the regeneration: microcytes were still present due to the injection and macrocytes occurred due to the newformation. During the regeneration phase normoblasts generally appeared in the blood.

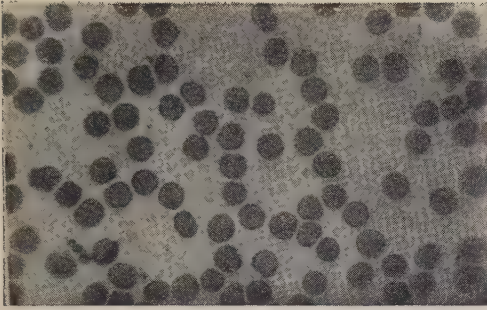


Fig. 2.
Before injection.

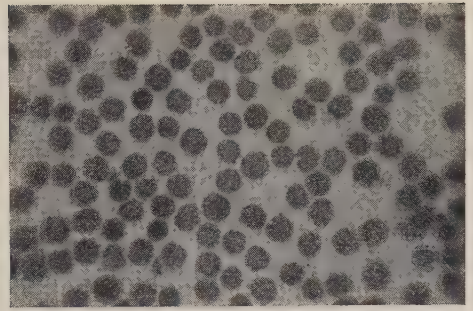


Fig. 3.
Three hours after injection. The erythrocyte diameter is somewhat smaller than before the treatment.

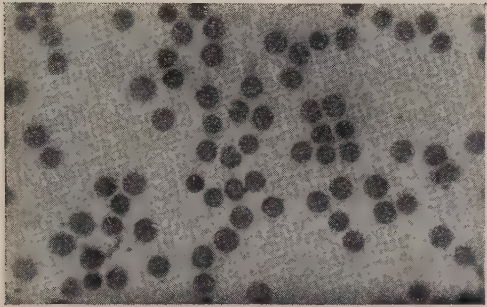


Fig. 4.
Two days after injection. Marked microspherocytosis.

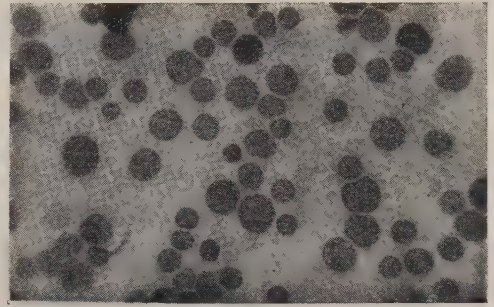


Fig. 5.
Five days after injection. The field contains microcytes, macrocytes, and one normoblast.

Figs. 2—5. Photomicrographs of smears prepared before and after intravenous injection of anti-erythrocyte serum. $\times 400$. Experiment No. 85.

Ten animals were given daily intraperitoneal injections of immune serum for four to seven days. These animals also acquired hemolytic anemia of the type described above. A decrease in the number of erythrocytes and a spherocytosis was observed — like after an intravenous injection — but these changes occurred later when the hemolysin was given intraperitoneally. The doses and their effect on the red blood picture are shown in Table 7. Half of the animals in this series died of hemolytic anemia. The remainder recovered and showed signs of increased regeneration of erythrocytes. An abundance of large polychromatic corpuscles were seen in the usual smears which were stained with May-Grünwald and Giemsa solutions, and a large number of reticulocytes occurred in the vitally stained preparations. The mean volume of the erythrocytes increased simultaneously.

Leucocyte counts were neither done in this nor in the previous experimental group, but the smears showed that the serum injections were followed by an appreciable leucocytosis.

TABLE 6.

CHANGES IN BLOOD VALUES AFTER INTRAVENOUS INJECTION OF ANTI-ERYTHROCYTE SERUM

No.	Serum No. and dilution	Dose/100gm. in ml. and H.U.		Before inj.	After injection								
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	
84	II 1:2	0.1 ml. 4 H.U.	R.B.C.	4.56	5.18	4.43	3.60	3.53		4.38		4.59	
			M.C.V.	95	85	81	100	105		96			
			M.C.D.	7.23	6.97	6.54	7.00	7.38		7.24			
			Micr. %	0	0.5	5.5	4.5	0.5		0.5			
			M.C.T.	2.3	2.3	2.4	2.6	2.6		2.3			
			Sph. I.	0.32	0.33	0.37	0.37	0.35		0.32			
85	»	0.15 ml. 6 H.U.	R.B.C.	5.29	5.10	3.81	2.40	1.82	1.70	2.17			After 9 d R.B.C. 4.46
			M.C.V.	84	78	77	77	94		106			
			M.C.D.	7.25	6.86	6.53	6.03	6.64		7.03			
			Micr. %	0	2.5	12	28.5	14		7.5			
			M.C.T.	2.0	2.1	2.3	2.7	2.7		2.7			
			Sph. I.	0.28	0.31	0.35	0.45	0.41		0.38			
87	»	»	R.B.C.	4.78	4.78	4.03	2.32	2.02	1.67	1.77		2.58	
			M.C.V.	92	89	79	71	79		121			
			M.C.D.	7.34	6.67	6.03	6.00	6.55		7.12			
			Micr. %	0	4	25	37	25		12			
			M.C.T.	2.2	2.5	2.8	2.5	2.3		3.0			
			Sph. I.	0.30	0.37	0.47	0.42	0.35		0.42			
88	»	»	R.B.C.	5.38	5.14	4.21	3.03	2.72	2.15	2.27		2.61	
			M.C.V.	89	88	81	83	79		90			
			M.C.D.	7.47	6.89	6.62	6.74	6.69		7.22			
			Micr. %	0	0.5	7	5.5	10.5		8			
			M.C.T.	2.0	2.4	2.4	2.3	2.2		2.2			
			Sph. I.	0.27	0.35	0.36	0.34	0.33		0.30			
94	»	»	R.B.C.	5.89	4.19	3.71	2.73	2.13	1.83	2.41			
			M.C.V.	78	76	74	73	80		91			
			M.C.D.	7.07	6.27	5.96	5.89	5.99		6.80			
			Micr. %	0.5	9	20.5	26	21.5		7.5			
			M.C.T.	2.0	2.5	2.6	2.7	2.8		2.5			
			Sph. I.	0.28	0.28	0.44	0.46	0.47		0.37			
138	»	»	R.B.C.	5.47	3.70	3.23	2.97	1.98	1.76	1.95	2.22		
89 spl.	»	»	R.B.C.	5.23	4.23	4.47	3.76	3.03	3.10	2.94	3.03	3.15	
			M.C.V.	87	84	78	70	76		95			
			M.C.D.	7.35	6.75	6.71	6.57	6.78		7.06			
			Micr. %	0.5	2.5	5	5.5	5		3			
			M.C.T.	2.0	2.3	2.2	2.1	2.1		2.4			
			Sph. I.	0.27	0.34	0.33	0.32	0.31		0.34			

TABLE 6 (continued)

No.	Serum No. and dilution	Dose/100gm. in ml. and H.U.		Before inj.	After injection								
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	
90 spl.	II 1:2	0.15 ml. 6 H.U.	R.B.C.	5.67	5.39	4.75	3.92	3.91	3.33	3.40		3.61	
			M.C.V.	82	75	79	72	65		82			
			M.C.D.	7.28	6.91	6.77	6.52	6.74		6.82			
			Micr. %	1.5	3	3	4	3.5		4.5			
			M.C.T.	2.0	2.0	2.2	2.2	1.8		2.2			
			Sph. I.	0.27	0.29	0.32	0.34	0.27		0.32			
91 spl.	»	»	R.B.C.	5.43	4.54	3.67	2.84	2.38	2.89	2.68		2.56	9 d
			M.C.V.	77	77	79	83	84		75			R.B.C.
			M.C.D.	7.27	6.78	6.59	6.61	6.83		7.01			2.99
			Micr. %	0	1.5	4	5	2		4			
			M.C.T.	1.9	2.1	2.3	2.4	2.3		1.9			
			Sph. I.	0.26	0.31	0.35	0.36	0.34		0.27			
97 spl.	»	»	R.B.C.	5.13	4.93	4.67	3.63	3.24	2.95	2.74		3.47	
			M.C.V.	89	83	72	78	77		89			
			M.C.D.	7.42	6.84	6.64	6.53	6.78		7.07			
			Micr. %	2.5	2	4	6	4.5		4			
			M.C.T.	2.1	2.3	2.1	2.3	2.1		2.3			
			Sph. I.	0.28	0.34	0.32	0.35	0.31		0.32			
139	II 1:1	0.15 ml. 12 H.U.	R.B.C.	5.08	2.88								Died in 24 hours
83	IV 1:4	0.1 ml. 1 H.U.	R.B.C.	5.91	5.71	5.90	5.41	5.01	5.13	4.88		4.93	
			M.C.V.	85	92	84	87	88		87			
			M.C.D.	7.25	6.98	7.10	7.03	7.09		7.09			
			Micr. %	0	0	0	0	0		0.5			
			M.C.T.	2.1	2.4	2.1	2.2	2.2		2.2			
			Sph. I.	0.29	0.34	0.30	0.31	0.31		0.31			
82	IV 1:2	0.1 ml. 2 H.U.	R.B.C.	5.82	6.09	5.44	5.16	4.65	4.05	4.03		5.00	
			M.C.V.	85	79	88	81	78		99			
			M.C.D.	7.27	7.29	7.13	7.04	7.02		7.23			
			Micr. %	0	0	0.5	0.5	0.5		0			
			M.C.T.	2.0	1.9	2.2	2.1	2.0		2.4			
			Sph. I.	0.27	0.26	0.30	0.30	0.28		0.33			
86	»	0.15 ml. 3 H.U.	R.B.C.	5.63	5.49	5.41	3.92	3.92	3.29	3.44			9 d
			M.C.V.	80	77	76	80	80		90			R.B.C.
			M.C.D.	6.92	6.82	6.82	6.55	6.88		6.86			4.32
			Micr. %	1	1	3	5	3		3.5			
			M.C.T.	2.1	2.1	2.1	2.4	2.1		2.4			
			Sph. I.	0.30	0.31	0.31	0.37	0.32		0.35			
136	»	»	R.B.C.	5.51	5.59	4.95	3.93	3.17	2.96	3.57			

TABLE 6 (continued)

No.	Serum No. and dilution	Dose/100gm. in ml. and H.U.		Before inj.	After injection									
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d		
137	IV	0.15 ml.	R.B.C.	5.92	5.84	4.53	2.27	1.27	1.19	1.76				
108	1:1	6 H.U.	R.B.C.	5.50	5.18	4.92	4.90	4.36	4.10	3.84		3.58	9 d	
	1:64	0.5 H.U.	M.C.V.	82	83	83	84	88	98	87			R.B.C.	
			M.C.D.	7.07	6.91	6.89	7.03	7.03	6.98	7.09			3.78	
			Micr.%	0.5	0.5	0	0	0	0					
			M.C.T.	2.1	2.2	2.2	2.2	2.3	2.6	2.2				
			Sph. I.	0.30	0.32	0.32	0.31	0.33	0.37	0.31				
107	V	0.1 ml.	R.B.C.	5.94	5.70	5.13	4.61	3.45	2.69	2.60		3.26	10 d	
	1:32	1 H.U.	M.C.V.	81	78	83	72	83	87	98			R.B.C.	
			M.C.D.	7.16	6.99	6.39	6.46	6.49	6.58	6.77			3.87	
			Micr.%	0	0.5	14	12.5	7	1	7				
			M.C.T.	2.0	2.0	2.6	2.2	2.5	2.6	2.7				
			Sph. I.	0.28	0.29	0.40	0.36	0.38	0.40	0.40				
112	V	0.15 ml.	R.B.C.	6.13	6.22	5.75	4.61	3.64	3.15	2.79	2.94	3.33		
	1:32	1.5 H.U.	M.C.V.	78	76	71	78	76	83	104		99		
			M.C.D.	7.34	7.10	6.71	6.39	6.41	6.40	6.64		6.87		
			Micr.%	0.5	0.5	4	11	8.5	5	5.5		4.5		
			M.C.T.	1.8	1.9	2.0	2.4	2.4	2.6	3.0		2.7		
			Sph. I.	0.25	0.27	0.30	0.38	0.37	0.41	0.45		0.39		
114	»	»	R.B.C.	4.52	3.88	3.74	2.65	2.19	2.41	2.64				
			M.C.V.	83	89	82	87	110	108	114				
			M.C.D.	7.05	6.91	6.33	6.33	6.37	6.95	7.23				
			Micr.%	0	1.5	18.5	23	29	14	10				
			M.C.T.	2.1	2.4	2.6	2.8	3.4	2.8	2.8				
			Sph. I.	0.30	0.33	0.41	0.44	0.53	0.40	0.39				
121 spl.	»	»	R.B.C.	6.21	5.21	4.94	3.92	3.27	2.79	2.99		3.39		
			M.C.V.	73	81	69	77	79	77	77		83		
			M.C.D.	6.99	6.91	6.45	6.26	6.38	6.73	6.95		7.23		
			Micr.%	2.5	2	15	18	11	6	5		2.5		
			M.C.T.	1.9	2.2	2.1	2.5	2.5	2.2	2.0		2.0		
			Sph. I.	0.27	0.32	0.33	0.40	0.39	0.33	0.29		0.28		
125 spl.	»	»	R.B.C.	5.11	5.47	5.17	5.18	4.45	4.68	4.90		4.94		
			M.C.V.	84	80	81	74	78	75	71		79		
			M.C.D.	7.27	7.04	6.93	6.88	6.93	7.11	7.19		7.33		
			Micr.%	0	1.5	2.5	4	1.5	0.5	0		4		
			M.C.T.	2.0	2.1	2.1	2.0	2.1	1.9	1.8		1.9		
			Sph. I.	0.28	0.30	0.30	0.29	0.30	0.27	0.25		0.26		

TABLE 7.

CHANGES IN BLOOD VALUES IN GUINEA-PIGS TREATED WITH DAILY
INTRAPERITONEAL INJECTIONS OF ANTI-ERYTHROCYTE SERUM

No.	Serum No. and dilution	Daily doses in ml./100 gm. Total dose in H.U.		Before treat- ment	After first injection						
					2 d	4 d	6 d	8 d	10 d	12 d	
58	II 1:4	0.1 ml. $\times$ 6 Total 12 H.U.	R.B.C.	4.71	4.29	2.29	1.12	1.47		2.74	
			M.C.V.	85	83	79	108	157			
			M.C.D.	7.25	6.91	6.67	7.25	8.01			
			Micr. %	0.5	1.5	1	4	6.5			
			M.C.T.	2.1	2.2	2.3	2.6	3.1			
			Sph. I.	0.29	0.32	0.34	0.36	0.39			
62	»	»	R.B.C.	5.51	4.22	3.10	1.64	2.34		3.03	
			M.C.V.	76	79	81	98	124			
			M.C.D.	6.98	7.07	6.68	7.60	7.73			
			Micr. %	0	0	2	0.5	2.5			
			M.C.T.	2.0	2.0	2.3	2.2	2.6			
			Sph. I.	0.29	0.28	0.34	0.29	0.34			
51 spl.	»	»	R.B.C.	4.08	4.65	3.81	3.41	2.80	3.42		
			M.C.V.	100	89	81	78	100			
			M.C.D.	7.54	7.52	7.35	7.36	7.25			
			Micr. %	0	0	0	0.5	0			
			M.C.T.	2.2	2.0	1.9	1.8	2.4			
			Sph. I.	0.29	0.27	0.26	0.24	0.33			
53 spl.	»	»	R.B.C.	5.06	4.92	4.27	3.39	2.43	2.90		
			M.C.V.	84	80	80	83	99			
			M.C.D.	7.61	7.39	7.43	7.32	7.47			
			Micr. %	0	0	0.5	0	0			
			M.C.T.	1.8	1.9	1.8	2.0	2.3			
			Sph. I.	0.24	0.26	0.24	0.27	0.31			
92	IV 1:2	0.1 ml. $\times$ 7 Total 14 H.U.	R.B.C.	6.40	5.58	4.97	4.07				After 7d R.B.C. 1.08, died in 8 d
93 spl.	»	»	R.B.C.	5.27	4.60	4.88	3.48	1.55	2.20		
109	V 1:16	0.1 ml. $\times$ 4 Total 8 H.U.	R.B.C.	5.84	4.92	0.47					Died after 4 d
			M.C.V.	77	85	96					
			M.C.D.	7.07	6.85	5.77					
			Micr. %	0	0	45					
			M.C.T.	2.0	2.3	3.7					
			Sph. I.	0.28	0.34	0.64					

TABLE 7 (continued)

No.	Serum No. and dilution	Daily doses in ml./100 gm. Total dose in H.U.		Before treat- ment	After first injection						
					2 d	4 d	6 d	8 d	10 d	12 d	
116	V 1:16	0.1 ml. × 4 Total 8 H.U.	R.B.C.	5.48	4.09	1.32					Died after 4 d
			M.C.V.	83	85	64					
			M.C.D.	6.91	6.56	6.53					
			Micr. %	0.5	2	58					
			M.C.T.	2.2	2.5	2.7					
			Sph. I.	0.32	0.38	0.49					
122 spl.	»	»	R.B.C.	5.35	5.82	3.29					Died after 4 d
			M.C.V.	86	77	71					
			M.C.D.	6.90	6.90	6.22					
			Micr. %	0	1.5	20					
			M.C.T.	2.3	2.1	2.3					
			Sph. I.	0.33	0.30	0.37					
123 spl.	»	»	R.B.C.	5.44	4.64	1.80					Died after 4 1/2 d
			M.C.V.	81	71	78					
			M.C.D.	7.10	6.50	6.10					
			Micr. %	0.5	16	42					
			M.C.T.	2.0	2.1	2.7					
			Sph. I.	0.28	0.32	0.44					

DIFFERENT DOSES AND DIFFERENT SERA. THE SIGNIFICANCE OF THE SPLEEN. ERYTHROPHAGOCYTOSIS

Quantitative comparison of the hemolytic effect of the same serum on different (non-splenectomized) animals showed that the hemolysis was almost proportional to the doses given, but some individual differences after an equal dose were observed. They were much smaller, however, than in my previous experimental series (Wasastjerna 1948 a).

But, comparing the effect of different sera and giving the doses in hemolytic units, it was seen that the action of serum V was stronger than that of serum II and serum IV in vivo. For instance, 2 H. U. of serum V injected intravenously hemolyzed more corpuscles than 6 H. U. of serum II. Even when the animals were given several intraperitoneal injections, the effect of serum V was stronger than that of serum II and serum IV. The effect of the latter two sera was about equal per hemolytic unit. The hemolytic unit content of the different sera was estimated, as stated in Chapter III, on the basis of their total hemolysis titer. On the other hand, if the doses were calculated on the basis of the visible hemolysis or the

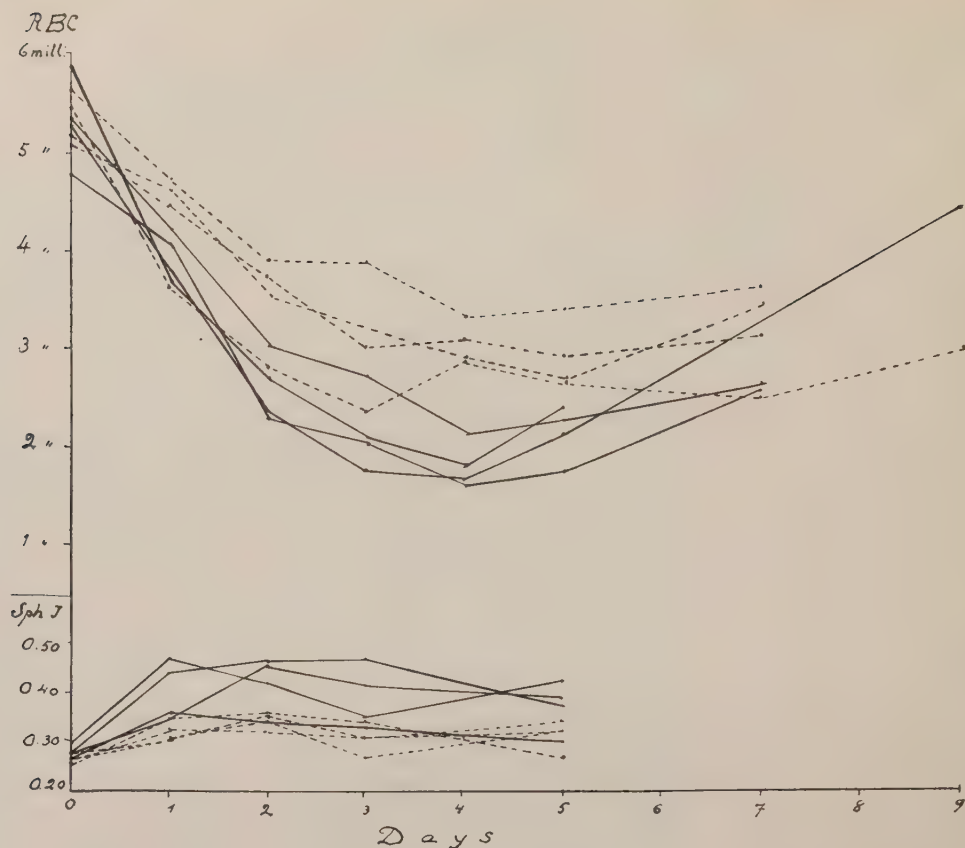


Fig. 6.

Erythrocyte count and spherical index in normal (unbroken lines) and splenectomized (broken lines) guinea-pigs after intravenous injection of 6 H. U. serum II per 100 gm. Experiments Nos. 85, 87, 88, 94, 89, 90, 91 and 97.

agglutinin titer, the action of serum V will still be found stronger than that of serum II and serum IV in vivo. This is best seen from Table 4 by comparing the titers of the different sera.

The significance of the spleen in experimental hemolytic anemia was studied in 13 parallel experiments on normal and splenectomized animals, i. e. eight with intravenous injections and five with repeated intraperitoneal injections. The results are given in Tables 6 and 7 in which the splenectomized animals have spl. after the animal's number, and in Table 8 in which the maximal changes in the erythrocyte counts, microcyte per cent, and spherical index have been worked out. For graphical clarity the results are also given in Figs. 6 to 10. These show the fall in number of erythrocytes and the increase in spherical index of the blood cells due to the treatment. The dotted lines show the changes in the splenectomized animals.

TABLE 8.
MAXIMAL CHANGES IN THE BLOOD VALUES IN PARALLEL EXPERIMENTS ON NORMAL AND
SPLENECTOMIZED ANIMALS

		Non-splenectomized animals				Splenectomized animals			
Experi- mental group	Dose/100 gm.	No.	Decrease in R.B.C. mill./cu. mm.	Increase in percentage of microcytes	Sph. I. Increase in % of initial value	No.	Decrease in R.B.C. mill./cu. mm.	Increase in percentage of microcytes	Sph. I. Increase in % of initial value
1.	6. H.U. of ser. II intraven.	85	3.59	28.5	61 %	89	2.29	5	26 %
		87	3.11	37	57 %	90	2.34	3	26 %
		88	3.23	10.5	33 %	91	3.05	5	38 %
		94	4.06	25.5	68 %	97	2.39	3.5	25 %
	Mean		3.50	25	54 %		2.52	4	29 %
2.	1.5 H.U. of ser. V intraven.	112	3.34	10.5	80 %	121	3.42	15.5	54 %
		114	2.33	29	77 %	125	0.66	4	7 %
	Mean		2.84	20	79 %		2.04	10	31 %
3.	2. H.U. of ser. V intraven.	115	>4.47 died	60.5	110 %	120	2.61	15	26 %
		111	4.30	27.5	54 %	124	2.96	21	25 %
	Mean		>4.39	44	82 %		2.79	18	26 %
4.	2 H.U. of ser. II × 6 intraperit.	58	3.59	6	34 %	51	1.28	0.5	14 %
		62	3.87	2.5	17 %	53	2.63	0.5	29 %
	Mean		3.73	4	26 %		1.96	0.5	22 %
5.	2 H.U. of ser. V × 4 intraperit.	109	>5.37 died	45	130 %	122	>2.06 died	20	12 %
		116	>4.16 died	57.5	53 %	123	>3.64 died	41.5	57 %
	Mean		?	51	92 %		?	31	35 %
6.	12 H.U. of ser. IV × 7 intraperit. Total 14 H.U.	92	>5.32 died			93	3.72		

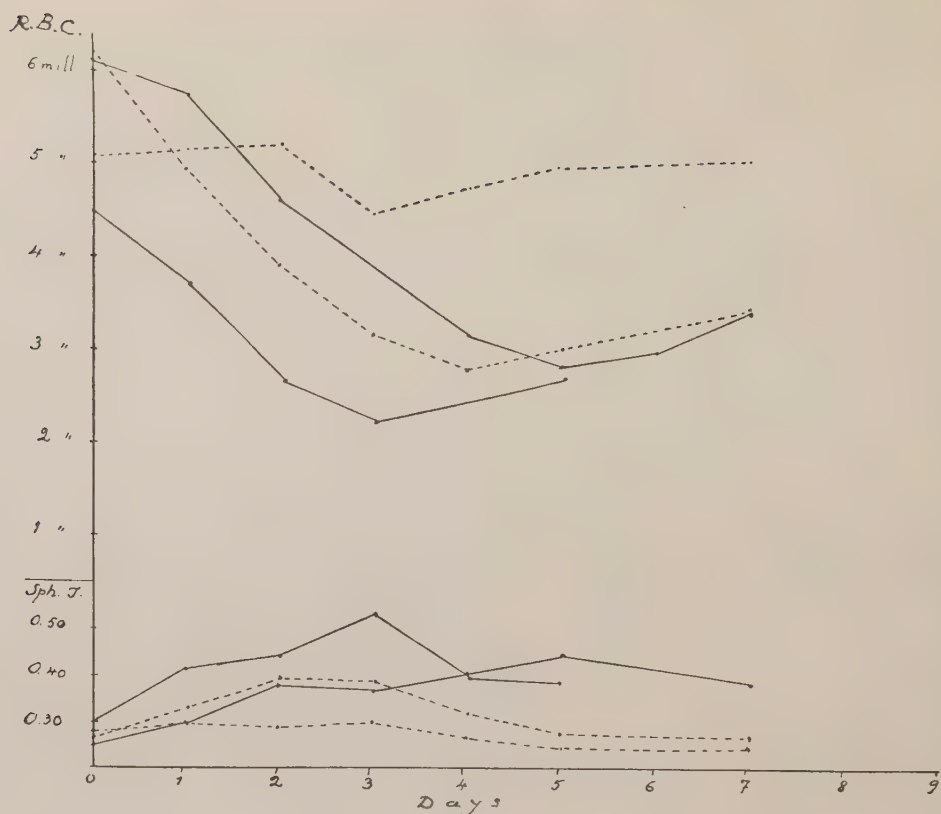


Fig. 7.

Erythrocyte count and spherical index after intravenous injection of 5 H. U. serum V.
Experiments Nos. 112, 114, 121 and 125.

The experiments were divided into six groups, each group being given different doses of anti-erythrocyte serum, i. e. the same dose per body-weight was given to all the animals within one group. The first group consisted of four normal and four splenectomized guinea-pigs and the last one of only one animal of each kind; in all the other groups there were two normal and two splenectomized animals. It is most distinctly seen from Table 8 that a certain amount of immune serum hemolyzes a much larger number of erythrocytes in animals with an intact spleen than in those who have had it removed. There was an exception in Group 2 only; in one of its splenectomized animals somewhat more erythrocytes were destroyed than in the normal animals, whereas, in another the decrease in the erythrocyte count was very small in comparison with that in the non-splenectomized guinea-pigs. In all the other groups the red blood count decreased more in the animals with a spleen. In Group 3 one of the two animals with an intact spleen died and

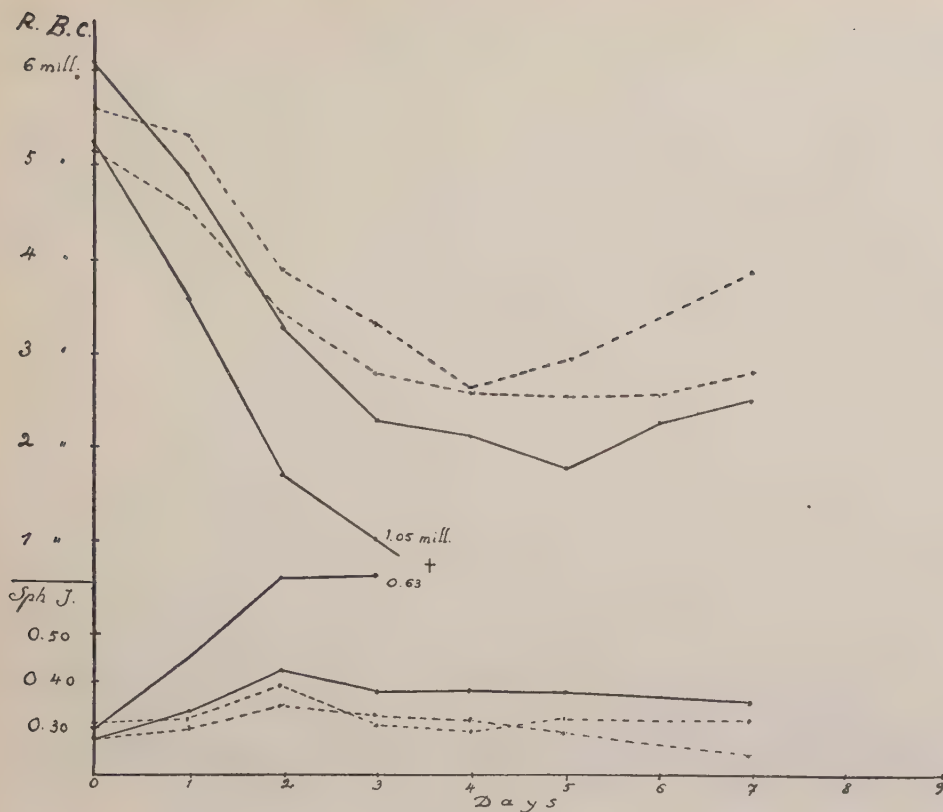


Fig. 8.

Erythrocyte count and spherical index after intravenous injection of 2 H. U. serum V.
Experiments Nos. 115, 111, 120 and 124.

both the splenectomized animals survived. Similarly in Group 6, the splenectomized animal survived. In Group 5 all animals died on the fifth day of the treatment, but four days after beginning the treatment the number of erythrocytes had decreased less in the splenectomized animals than in the others and they lived about 12 hours longer. The results may be considered definite in spite of the smallness of the material, i. e. a certain amount of anti-erythrocyte serum can in most cases dissolve more erythrocytes in normal animals than in those who have had their spleen removed.

In these experiments the change in shape of the non-dissolved erythrocytes was proportional to the hemolysis. Table 8 and the diagrams show distinctly that the spherical index of the red cells and the percentage of microcytes increased much more in the animals with an intact spleen than in those subjected to splenectomy.

In most of the nine splenectomized guinea-pigs who died of hemolytic anemia

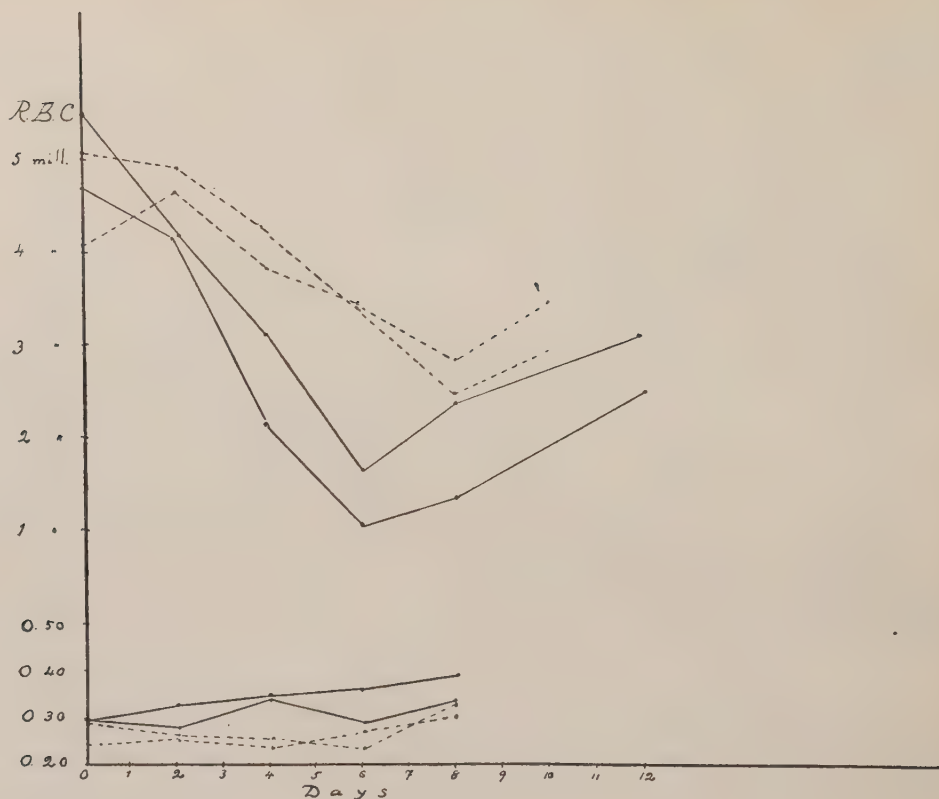


Fig. 9.

Erythrocyte count and spherical index in guinea-pigs given daily intraperitoneal injections of 2 H. U. serum II for 6 days. Experiments Nos. 58, 62, 51 and 53.

a somewhat enlarged spleen was observed on post mortem examination. The weight of the spleen varied between 0.8 and 1.6 gm., average 1.2 gm. For comparison it may be mentioned that after splenectomy the weight of 16 normal spleens ranged between 0.35 and 1.1 gm., average 0.6 gm. This is not, however, good comparative material as the spleens were taken from living guinea-pigs under anesthesia, hence the degree of enlargement could not be closely assessed.

Sections of the spleens stained with eosin and hematoxylin of six guinea-pigs who died of hemolytic anemia were examined microscopically. In all these preparations there were numerous red corpuscles in the pulp and here and there large cells containing erythrocytes (Fig. 11). Erythrophagocytosis was not observed in the preparations of two normal spleens. Still more distinct erythrophagocytosis was observed in the smears of spleens from two animals with severe hemolytic anemia than in the section preparations; one of these was made after the death of the animal from anemia, whereas the second animal (No. 141) was killed when the erythrocyte

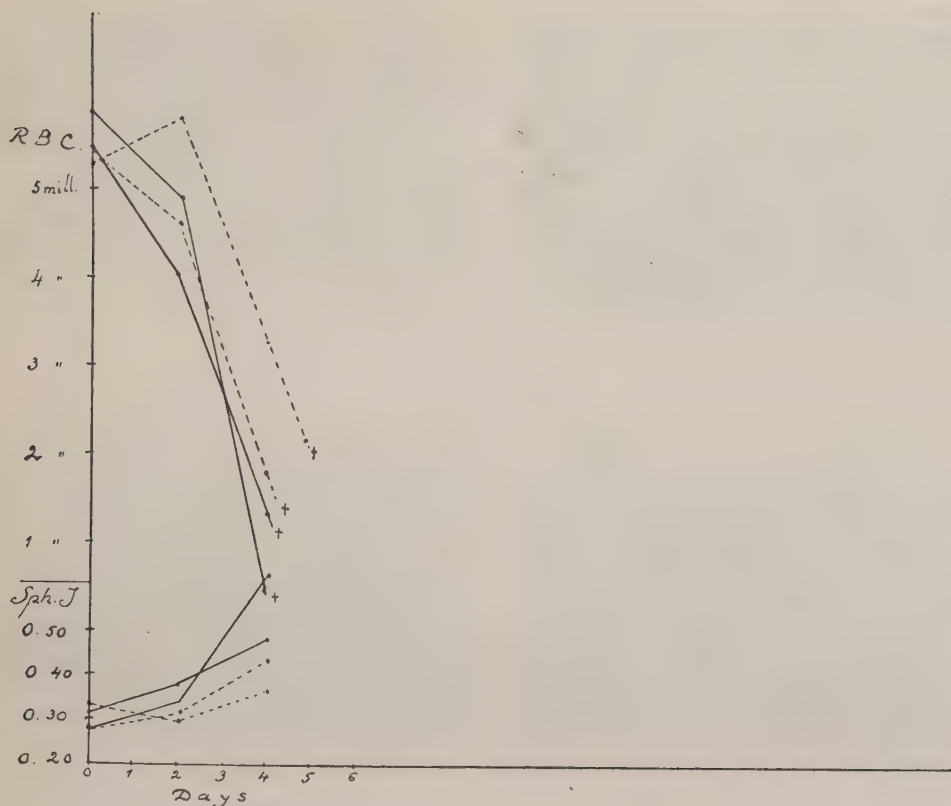


Fig. 10.

Erythrocyte count and spherical index in guinea-pigs given daily intraperitoneal injections of 2 H. U. serum V for 4 days. Experiments Nos. 109, 116, 122 and 123.

count had fallen to 0.59 millions and the smear of its spleen was immediately prepared. This preparation (Fig. 12) contained an abundance of cells which had ingested erythrocytes. This shows that the erythrophagocytosis was not a post mortem phenomenon in these cases.

Erythrophagocytosis was also seen in the peripheral blood in the form of rather large monocytes which had ingested one, or in exceptional cases two red corpuscles (Figs. 13—14). This kind of cell was very rare, however. A systematical study of blood smears from several animals with severe hemolytic anemia showed solitary erythrophagocytes in some, whereas in others there were none. That there actually was phagocytosis of red corpuscles and not a piling up of cells in the smears was proved by the erythrocytes in question not staining so well as the free erythrocytes and by these cells being often surrounded by a clear zone in the plasma of the phagocytizing cell. Erythrophagocytosis was never observed in blood smears of untreated guinea-pigs.

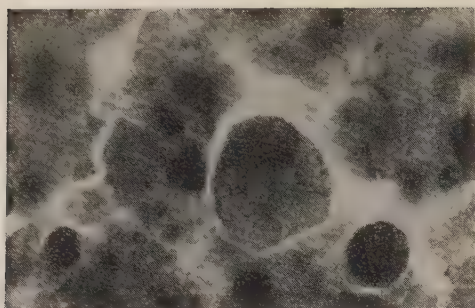


Fig. 11.

Macrophage containing red blood corpuscles in section of spleen.

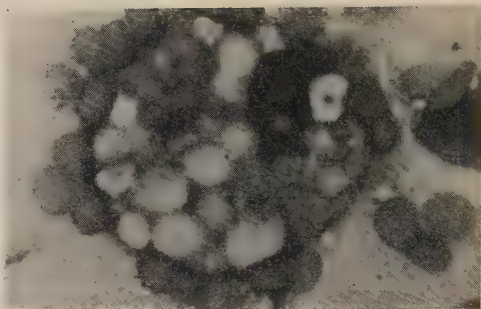


Fig. 12.

Macrophage containing red blood corpuscles in smear of spleen.

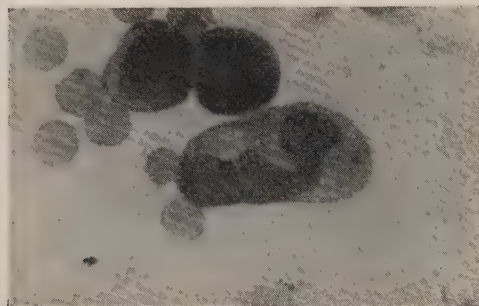


Fig. 13.

Smear with a leucocyte which has ingested two erythrocytes.

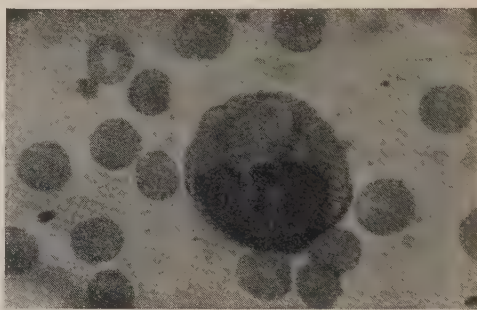


Fig. 14.

Smear with a monocyte which has ingested one erythrocyte.

Figs. 11-14. Phagocytosis of red blood cells in experimental hemolytic anemia. $\times 800$.

HEMAGGLUTINATION

Agglutination of cells in samples taken after injection of immune serum was always observed providing the dose had been large enough to induce distinct anemia. In the experiments described in Table 6, agglutination was observed in all the cases, except in Nos. 83, 82 and 108. In Case 84 there was some doubt as to the hemagglutination and in the non-treated animals it was never observed. Agglutination was found in the first samples taken three hours after the intravenous injection and then in all samples taken during three to five days, after which it was absent. On the fifth day after the injection it was always weak, if at all present. The method used was qualitative in character but my definite impression is that the agglutination was stronger one to two days after the injection than three hours after it. Also the animals treated with intraperitoneal injections of immune serum showed hemagglutination. This was observed in all the experiments mentioned in Table 7.

The problem next in order was naturally to find out whether agglutination of blood cells was a phenomenon occurring only *in vitro*, or was it previously present *in vivo*? When a drop of blood was taken from the animal treated and mixed with a drop of citrate solution on a slide, agglutination was not immediately observed macroscopically — it did not occur until half a minute or one minute later, being most distinct after three to five minutes. For that reason one small drop of blood was taken on a cover glass that was placed with the drop downward on a slide; no citrate solution was added. The drop was studied microscopically as soon as possible after being drawn. It was then found that distinct agglutination occurred in the drop of blood already 10 seconds after escaping from the ear vein. It was impossible to place the sample under the microscope in less than 10 seconds. The »rubber band phenomenon», described by Bessis and Freixa (1947), was distinctly seen, i. e. some of the cells formed a chain, their edges touching one another, i. e. not as rouleaux. When the chain was stretched, the erythrocytes became spindle-shaped, lying point to point but often slightly apart and joined by some invisible substance, until it broke up and the corpuscles reassumed their circular shape.

It was still not definitely known whether agglutination occurred also intravasally. For this purpose the flowing blood in the animal's vessels had to be observed. This was done with the aid of a binocular preparation microscope. The animals were given urethane and ether anesthesia and the blood vessels in the conjunctiva sclerae were studied at a magnification of 60 under oblique light. Two untreated guinea-pigs and two which had been given an intravenous injection of physiological salt solution were first examined. In these an insignificant aggregation of the corpuscles in the blood stream was observed and the circulating blood seemed to be finely granulated. This was perhaps caused by the anesthesia. The results were similar in two animals given intravenous injections of saponin. But two guinea-pigs, given an intravenous injection of 2 H. U. per 100 gm. of serum V, showed, on the following day, a very strong intravascular agglutination visible both in the veins and the arteries. The small vessels often seemed to be empty for some distance along their course, and only solitary large agglomerations of erythrocytes were seen to move rather slowly through the vessel, stopping occasionally for a second or two in a narrow part where they were gradually pressed together from the sides and drawn out lengthwise to enable them to pass and to continue their course. In the larger vessels the whole blood stream consisted of large or small agglomerations.

SUMMARY OF CHAPTER V

Guinea-pigs given one intravenous or repeated intraperitoneal injections of immune serum (anti-erythrocyte serum) acquired hemolytic anemia, the red blood picture being micro-spherocytic. The erythrocyte count decreased during approx-

imately four days after an intravenous injection, while the spherocytosis was most pronounced about two days after the injection. Three hours after the injection the erythrocyte count had only slightly decreased and the change in shape was very small.

The effect of different sera *in vivo* was not directly proportional to their titer *in vitro*.

A certain amount of immune serum did not dissolve as many red corpuscles in the splenectomized animals as in those with an intact spleen. The spherocytosis was proportional to the hemolysis, thus being less pronounced in the blood of guinea-pigs whose spleen had been removed.

An intense phagocytosis of red cells was observed microscopically in the spleens of the animals treated and very sparce erythrophagocytosis in the blood smears of some of the animals with severe hemolytic anemia.

Providing the amount of immune serum was sufficient to induce distinct hemolytic anemia, agglutination of the erythrocytes occurred in the experimental animals' blood. Capillary microscopy showed that the agglutination was intravasal.

CHAPTER VI

ANIMAL EXPERIMENTS WITH SAPONIN, STREPTOLYSIN AND BEAN EXTRACT

EXPERIMENTS WITH SAPONIN

In the first preliminary experiments with saponin, low concentrations were used in order that the hemolysin titer should be about the same as the titers of those immune serum dilutions necessary to induce a severe hemolytic anemia. Since no hemolytic effect resulted *in vitro* the concentration was gradually increased, 20 per cent solutions being used in the final experiments. They were given intravenously in doses ranging from 0.1 to 0.32 ml. per 100 gm. body-weight. As the hemolysin titer of this saponin solution was as high as 1:1 536, the doses given were 256 to 768 H. U. per 100 gm. That they were effective *in vivo* is proved by hemoglobin being found in the urine of all the experimental animals; it was always observed in the first portion passed after the injection. Sometimes hemoglobinuria occurred already during the injection but never one day after. Urobilin, but never hemoglobin, was often found in the urine on the second or third day.

Whereas the hemoglobinuria showed that the doses of saponin given had an immediate hemolytic effect *in vivo*, the effect on the number of red cells was relatively moderate and rather variable. No appreciable anemia was induced in any of the animals. The doses could not be increased, however, as their effect was markedly toxic. Some of the animals died although the fall in the erythrocyte count was small. If the injections were given rapidly, the animals died immediately. Since the cause of death was not anemia, only the surviving animals, a total of 11, are here included. Table 9 shows the influence of the doses on the red corpuscles. In two cases a decrease of over 2 millions per cu. mm. was observed, in six cases 1 to 2 millions, and in three under 1 million. In the majority the blood count decreased most on the first day while the lowest erythrocyte count was recorded after greatly varying periods in the different experiments, between three hours and five days after the termination of the injection, but mostly after three to four days. When the decrease in number of erythrocytes was not very small, the number of reticulocytes increased five days after the injection in some cases but not in all.

The mean volume of the erythrocytes varied slightly in some of the animals,

TABLE 9.
EXPERIMENTS WITH INJECTIONS OF SAPONIN

No.		Dose/100gm. in ml. and H.U.		Before inj.	After injection								
					3h	1d	2d	3d	4d	5d	6d	7d	
65	20 % saponin solution	0.1 ml. 256 H.U.	R.B.C.	5.54	5.52	4.82	4.47	4.05		4.10			9 d R.B.C. 4.49
			M.C.V.	81	79	78	85	93		95			
			M.C.D.	7.34	7.14	7.47	7.39	7.37		7.55			
			Micr. %	0	0	0	0	0.5		0			
			M.C.T.	1.9	2.0	1.8	2.0	2.2		2.1			
			Sph. I.	0.26	0.28	0.24	0.27	0.30		0.28			
80	»	»	R.B.C.	5.34	5.15	5.18	5.21	5.27	5.19	4.99		5.90	
			M.C.V.	86	88	90	92	84		90			
			M.C.D.	7.33	7.31	7.33	7.35	7.30		7.12			
			Micr. %	0	0.5	0	0	0		0			
			M.C.T.	2.0	2.1	2.1	2.2	2.0		2.3			
			Sph. I.	0.27	0.29	0.29	0.30	0.27		0.32			
79 spl.	»	»	R.B.C.	5.25	5.39	4.76	4.96	4.72	4.56	4.56		4.58	
			M.C.V.	82	80	86	81	78		83			
			M.C.D.	7.36	7.22	7.41	7.20	7.28		7.30			
			Micr. %	0.5	1.5	0.5	0	0.5		1.5			
			M.C.T.	1.9	2.0	2.0	2.0	1.9		2.0			
			Sph. I.	0.26	0.28	0.27	0.28	0.26		0.27			
34	»	0.2 ml. 512 H.U.	R.B.C.	5.97	5.15	4.53	4.33	3.63	3.64	3.60		5.03	
			M.C.V.	80	79	79	82	85		89			
			M.C.D.	7.19	7.05	7.14	7.01	6.98		7.15			
			Micr. %	0.5	0.5	0	1	0.5		0			
			M.C.T.	2.0	2.0	2.0	2.1	2.2		2.2			
			Sph. I.	0.28	0.28	0.28	0.30	0.32		0.31			
73	»	»	R.B.C.	5.52	4.27	4.89	4.79	5.22		4.43		4.47	
			M.C.V.	81	97	81	87	80		90			
			M.C.D.	7.20	6.94	7.28	7.29	7.33		7.18			
			Micr. %	0	2	0.5	0.5	0		0			
			M.C.T.	2.0	2.6	1.9	2.1	1.9		2.2			
			Sph. I.	0.28	0.37	0.26	0.29	0.26		0.31			
76 spl.	»	»	R.B.C.	5.86	5.11	4.97	4.83	4.26	4.61	4.30		4.61	
			M.C.V.	82	93	82	84	92		90			
			M.C.D.	7.34	7.27	7.16	7.28	7.34		7.43			
			Micr. %	0.5	1.5	0.5	0.5	0.5		1.0			
			M.C.T.	1.9	2.2	2.0	2.0	2.2		2.1			
			Sph. I.	0.26	0.30	0.28	0.27	0.30		0.28			

TABLE 9 (continued)

No.		Dose/100gm. in ml. and H.U.		Before inj.	After injection							
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d
27 spl.	20 % saponin solution	0.2 ml. 512 H.U.	R.B.C.	5.52	5.37	5.60	5.89	5.39	5.43	5.12		5.40
			M.C.V.	81	83	77	76	74		78		78
			M.C.D.	6.96	6.72	6.70	6.70	6.83		6.83		6.99
			Micr. %	2.5	6.5	4.5	6.5	3.5		5.5		3
			M.C.T.	2.1	2.3	2.2	2.2	2.0		2.1		2.0
			Sph. I.	0.30	0.34	0.33	0.33	0.29		0.31		0.29
35	»	0.3 ml. 768 H.U.	R.B.C.	6.19	6.07	5.80	5.95	5.23	5.17	5.98		
			M.C.V.	78	77	76	73	74		73		
			M.C.D.	7.40	7.06	6.71	6.96	6.70		6.74		
			Micr. %	0	1	2	0	0.5		0.5		
			M.C.T.	1.8	2.0	2.1	1.9	2.1		2.0		
			Sph. I.	0.24	0.28	0.31	0.27	0.31		0.33		
37	»	»	R.B.C.	5.32	4.72	4.43	4.67	4.64	4.46	4.55		8 d R.B.C. 5.20
			M.C.V.	86	85	81	85	80		87		
			M.C.D.	7.25	6.94	7.05	6.90	6.90		7.17		
			Micr. %	0.5	0.5	3	2	3.5		0.5		
			M.C.T.	2.1	2.2	2.1	2.3	2.1		2.1		
			Sph. I.	0.29	0.32	0.30	0.33	0.30		0.29		
23 spl.	»	»	R.B.C.	5.61	4.47	4.12	4.06	3.61	4.01	4.17		4.81
			M.C.V.	72	65	63	64	68		68		
			M.C.D.	6.88	6.65	6.45	6.57	6.73		6.94		
			Micr. %	7	8.5	11.5	8.5	8		7		
			M.C.T.	1.9	1.9	1.9	1.9	1.9		1.8		
			Sph. I.	0.28	0.29	0.29	0.29	0.29		0.26		
36	»	0.32 ml. 819 H.U.	R.B.C.	6.27	6.07	4.15	5.01	4.48	4.53	4.83		12 d R.B.C. 5.48
			M.C.V.	75	77	84	73	83		80		
			M.C.D.	7.00	6.80	6.48	6.39	6.53		6.72		
			Micr. %	0	1	2.5	3	2.5		0.5		
			M.C.T.	2.0	2.1	2.5	2.3	2.5		2.2		
			Sph. I.	0.29	0.31	0.39	0.36	0.38		0.33		

but there was no regular increase or decrease in the entire series. As it is known that the saponin hemolysis in vitro is preceded by spherocytosis it might be thought that the shape of the erythrocytes would be influenced although their number was not greatly affected. A change in shape was actually observed but it was not so marked as might have been expected. The percentages of the microcytes increased by only 0.5 to 4.5 and the spherical index of the erythrocytes increased in the animal

given the largest dose, from 0.29 to 0.39, or 34 per cent. In all the other experiments the increase in the spherical index was less, on an average only 0.05, or 17 per cent of the initial value. There was a moderate increase in the spherical index of the corpuscles in all the experiments, hence it is statistically significant although the mean was somewhat less than 2.58 times the standard deviation in the control experiments (Chapter IV). The increase was observed in all cases except one after only three hours; by this time the maximum had been reached in five of the eleven tests. In the remaining cases the maximum was attained in one to five days. The largest number of microcytes was also observed in five cases already after three hours, and in the others in one to three days. In two experiments (Nos. 65 and 80) a smear was made immediately after the injection in order to discover whether a larger number of microspherocytes had been dissolved already after three hours. This was not the case. No microcytes, i. e. corpuscles with a diameter of $5\ \mu$ or less were found in these two smears.

Four of the 11 experimental animals had previously been splenectomized. They are designated spl. in Table 9. The effect of the saponin injections on the number and shape of the red blood cells in the animals without a spleen did not differ from that in other animals. These parallel tests, so few in number, do not allow conclusions regarding very small quantitative variations. A much larger series of experiments on normal and splenectomized animals was first planned, but the idea was abandoned as it was found that saponin did not induce true hemolytic anemia.

EXPERIMENTS WITH STREPTOLYSIN

Five guinea-pigs were given intravenous injections of undiluted streptolysin with the titer 1:96 or 1:192, in doses of 0.5, 0.75, or 1.0 ml. per 100 gm. body-weight. If the doses are given in hemolytic units, their sizes would be 120, 160 or 240 H. U. per 100 gm. respectively. The streptolysin was detrimental to the animal's general condition and had to be injected very slowly in order to avoid a fatal shock. With the doses mentioned it did not seem to be so toxic as the saponin injections. Like these, the streptolysin injections caused immediate hemoglobinuria which was observed during the injection or as soon as urine was discharged after it. The hemoglobinuria ceased during the first day after the injection. Streptolysin O thus has an immediate hemolytic effect when injected intravenously in adequate doses.

Anemia was not induced by streptolysin injections. As seen from Table 10, the erythrocyte count fell slightly, but the greatest decrease observed in one case was only 1.60 millions per cu. mm.; in the others it was still less. In all the tests the decrease in the number of erythrocytes occurred mainly during the first day after the injection.

The shape of the erythrocytes did not change in most of the experiments. The spherical index increased distinctly in one case only (No. 30), i. e. from 0.27 to

TABLE 10.
EXPERIMENTS WITH INJECTIONS OF STREPTOLYSIN

No.	Streptolysin No. and titer	Dose/100gm. in ml. and H.U.		Before treat- ment	After (first) injection								
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	
38	Strepto- lysin III (titer 1:96)	0.75 ml.	R.B.C.	5.80	6.07	4.74	5.55	5.62		5.84			
			M.C.V.	81	82	94	87	80		80			
		120 H.U.	M.C.D.	7.42	7.51	7.37	7.41	7.28		7.44			
			Micr.%	0	0	0	0	0.5		0			
		intraven.	M.C.T.	1.9	1.8	2.2	2.0	1.9		1.8			
			Sph. I.	0.26	0.24	0.30	0.27	0.26		0.24			
40	»	1 ml.	R.B.C.	5.37	5.41	4.75	5.01	5.00		5.05			9d R.B.C. 5.31
			M.C.V.	80	80	84	82			84			
		160 H.U.	M.C.D.	7.21	7.17	7.04	7.05			7.14			
			Micr.%	0	0.5	0	0			0			
		intraven.	M.C.T.	2.0	2.0	2.2	2.1			2.1			
			Sph. I.	0.28	0.28	0.31	0.30			0.29			
135	Strepto- lysin V (titer 1:192)	0.5 ml.	R.B.C.	5.40	5.14	4.47	4.40	4.51		5.02			
			M.C.V.	88	94	82	83	87		92			
		160 H.U.	M.C.D.	7.08	6.98	6.88	7.02	7.17		7.35			
			Micr.%	0.5	0.5	2.5	1.5	0.5		1			
		intraven.	M.C.T.	2.2	2.5	2.2	2.1	2.2		2.2			
			Sph. I.	0.31	0.36	0.32	0.30	0.31		0.30			
30	Strepto- lysin II (titer 1:192)	0.5 ml.	R.B.C.	6.55	5.86	5.49	5.46	5.21		6.05			
			M.C.V.	73	75	77	76	76		81			
		160 H.U.	M.C.D.	6.97	7.11	6.47	6.67	6.79					
			Micr.%	1	0.5	7	3.5	3.5					
		intraven.	M.C.T.	1.9	1.9	2.3	2.2	2.1					
			Sph. I.	0.27	0.27	0.36	0.33	0.31					
32	»	0.75 ml.	R.B.C.	5.49	4.79	3.97	3.89	4.02			4.46		
			M.C.V.	83	77	82	77	80			87		
		240 H.U.	M.C.D.	7.14	7.10	7.12	7.11	7.14			7.21		
			Micr.%	1	1.5	0	1	0			0		
		intraven.	M.C.T.	2.1	1.9	2.0	1.9	2.0			2.1		
			Sph. I.	0.29	0.27	0.28	0.27	0.28			0.29		
39	Strepto- lysin III	1.0 ml. × 6	R.B.C.	5.45			5.86		5.79		5.58		9d R.B.C. 5.90
			M.C.V.	84			79		80		80		
		intraperit.	M.C.D.	7.12			7.17		7.23		6.95		
			Micr.%	0			0		0		0.5		
		Total 960 H.U.	M.C.T.	2.1			2.0		1.9		2.1		
			Sph. I.	0.30			0.28		0.26		0.30		

0.36, and the microcytes increased in number from 1 to 7 per cent. In another case (No. 135) the index increased so little that there was some doubt whether it was due to a specific effect of the streptolysin. In the other experiments the spherical index of the cells varied somewhat, it is true, but not more than in the control experiments (Chapter IV), hence spherocytosis cannot be considered in these cases.

One of the guinea-pigs (No. 39) was given large intraperitoneal doses of streptolysin, 1 ml. per 100 gm. daily for six days, or a total of 960 H. U. per 100 gm. Hemoglobinuria did not occur, nor did the erythrocyte count decrease or the spherical index increase.

EXPERIMENTS WITH BEAN EXTRACT

Three guinea-pigs were given intravenous injections of bean extract: in two cases an undiluted extract with the agglutinin titer 1:2 400 in doses of 0.1 and 0.2 ml. per 100 gm. body-weight, and in one case 0.3 ml. per 100 gm. of an extract with the titer 1:3 072. The agglutinating effect of the extracts, which were prepared in exactly the same way, was probably equally strong but the former was diluted 1:100 before titration, hence the dilutions in the test tubes and, in consequence, the titer values obtained varied slightly. As seen from Table 11, there was a very small decrease in the red cell count in all the three experiments one to two days after the injection. As the decrease was only 0.67, 0.51 and 0.58 millions per cu. mm., i. e. 12.5, 9.7 and 10.9 per cent of the initial value and as three days after the injection the count had increased to the initial value without reticulocytosis occurring the injection had probably caused no destruction of erythrocytes. There was no hemoglobinuria in the urine after the injection but Schlesinger's urobilin test was slightly positive in experiment No. 42 after two days and in experiment No. 46 after one day. However, it is not certain whether the urobilinuria was due to hemolysis in the guinea-pig. These three experiments thus show that intravenous injection of bean extract in the doses mentioned probably does not cause erythrocyte destruction in the experimental animals, but slight hemolysis cannot be wholly excluded. No hemagglutination was found in the blood samples taken after the injection. The spherical index of the red cells did not increase, but a slight decrease was observed in experiment No. 46. In the same experiment there were 3.5 per cent microcytes three hours after the injection, but this is hardly of any importance.

Guinea-pig No. 63 was given daily intraperitoneal doses of 0.1 ml. of bean extract with the titer 1:3 072 for six days. The erythrocyte count which was 6.38 millions before the first injection fell to 4.22 millions one day after the last injection and the spherical index of the corpuscles increased during the same period from 0.27 to 0.34. After a further two days the erythrocyte count fell to 3.94, thus decreasing by 2.44 millions in eight days, a decrease which is many times greater than the variations in the controls. No hemoglobinuria or urobilinuria occurred in this case, hence it cannot be definitely determined whether the decrease in

TABLE 11.
EXPERIMENTS WITH INJECTIONS OF BEAN EXTRACT

No.	Extr. No. and titer	Dose/100gm.		Before treat- ment	After (first) injection									
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d	
42	Extr. I titer 1:2 400	0.1 ml. intraven.	R.B.C.	5.34	5.22	4.67	5.12	5.50		5.19		4.80		
			M.C.V.	96	96	90	86	86		87				
			M.C.D.	7.58	7.49	7.70	7.47	7.43		7.47				
			Micr. %	0	0	0	0	0		0				
			M.C.T.	2.1	2.2	1.9	1.9	2.0		2.0				
			Sph. I.	0.28	0.29	0.25	0.25	0.27		0.27				
41	»	0.2 ml. intraven.	R.B.C.	5.14	5.52	4.90	4.63	5.03		5.23		5.60		
			M.C.V.	93	86	82	87	84		83				
			M.C.D.	7.60	7.45	7.35	7.16	7.41		7.42				
			Micr. %	0	0	0	0	0		0.5				
			M.C.T.	2.0	2.0	1.9	2.0	1.9		1.9				
			Sph. I.	0.26	0.27	0.26	0.28	0.26		0.26				
46	Extr. II titer 1:3 072	0.3 ml. intraven.	R.B.C.	5.32	5.30	4.94	4.74	5.29		5.28		5.64		
			M.C.V.	83	75	81	77	76		81				
			M.C.D.	6.98	6.70	7.04	7.15	7.34		7.34				
			Micr. %	0	3.5	0	0	0		0				
			M.C.T.	2.2	2.1	2.1	1.9	1.8		1.9				
			Sph. I.	0.32	0.31	0.30	0.27	0.25		0.25				
63	Extr. IV titer 1:3 072	0.1 ml. × 6 intraperit.	R.B.C.	6.38			6.22		5.94		4.22	3.94	12d R.B.C. 5.45	
			M.C.V.	77			76		77		105			
			M.C.D.	7.10			7.07		6.97		7.29			
			Micr. %	0			0		1.5		0			
			M.C.T.	1.9			1.9		2.0		2.5			
			Sph. I.	0.27			0.27		0.29		0.34			
133	Extr. V titer 1:3 072	0.1 ml. × 6 intraperit.	R.B.C.	4.71			4.56		4.48		4.64	4.75		
			M.C.V.	87			88		90		85	86		
			M.C.D.	7.33			7.00		7.16		7.35	7.35		
			Micr. %	0			0.5		0		0	0		
			M.C.T.	2.0			2.3		2.2		2.0	2.0		
			Sph. I.	0.27			0.33		0.31		0.27	0.27		
134	»	0.2 ml. × 6 intraperit.	R.B.C.	5.46			5.15		5.37		5.03	5.42		
			M.C.V.	80			85		83		79	74		
			M.C.D.	6.85			6.80		6.90		6.94	7.07		
			Micr. %	0			0.5		0		1	0.5		
			M.C.T.	2.2			2.3		2.2		2.1	1.9		
			Sph. I.	0.32			0.34		0.32		0.30	0.27		

number of erythrocytes was caused by a specific destructive effect of the bean extract or not. The absence of reticulocytosis argues against such a specific destructive effect. The reticulocyte count was only 0.7 per cent on termination of the treatment. The experiment was repeated with another extract with the same titer. An equal dose was given to one animal and twice as much to another (Nos. 133 and 134) without any effect on the red cell count or the spherical index resulting. Also these experiments are shown in Table 11.

Thus, a constant erythrocyte destruction was not obtained by intraperitoneal injections of bean extract. Larger doses were tried but the guinea-pigs were not able to stand them. Their abdomens distended and they died in a few days. Agglutination of red blood corpuscles was not observed on intraperitoneal injections of bean extract.

EXPERIMENTS WITH BEAN EXTRACT IN COMBINATION WITH SAPONIN AND STREPTOLYSIN

The experiments with saponin and streptolysin showed that these two substances have an immediate hemolyzing effect *in vivo* if the injections are given intravenously. The decrease in the erythrocyte count was small in comparison with the decrease after injection of immune serum although the doses were much larger expressed in hemolytic units.

Besides hemolysin, the immune sera used contained agglutinin. However, as agglutinin in the form of bean extract had no definite hemolytic effect alone *in vivo*, the question must be asked whether the strong effect of immune serum *in vivo* was not due to the combination of hemolysin and agglutinin. Experiments were therefore made with bean extract in combination with saponin in two cases and in combination with streptolysin in two. The substances were not mixed, but given intravenously immediately after each other, one of the animals being given first bean extract and then saponin, the second one first saponin and then bean extract, the third one first bean extract and then streptolysin and the fourth the latter substances in the reverse order. The doses and their action on the blood are shown in Table 12. It is seen that the hemolytic effect was not stronger than when saponin or streptolysin were given alone. That intravascular hemolysis followed upon the injections is evidenced by the hemoglobinuria in all the experiments, whereas no decrease in the erythrocyte count worth mentioning occurred in these cases. Injection of bean extract alone did not cause hemagglutination as stated, nor did it occur after injection of bean extract and saponin or streptolysin.

SUMMARY OF CHAPTER VI

Large doses of saponin solution injected intravenously into guinea-pigs induced immediate hemolysis with hemoglobinuria and a moderate or slight decrease in

TABLE 12.

EXPERIMENTS WITH INJECTIONS OF BEAN EXTRACT AND SAPONIN OR STREPTOLYSIN

No.	Substances injected	Dose/100gm.		Before inj.	After injection								
					3 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d	8 d
43	Bean extract I (titer 1:2 400) +	0.1 ml.	R.B.C.	4.97	5.36	4.04	4.83	4.56	5.01	4.23		4.27	
			M.C.V.	81	79	81	74	74		84			
			M.C.D.	7.55	7.26	7.23	7.15	7.30		7.23			
	20 % saponin (titer 1:1 536)	0.1 ml. (256 H.U.)	Micr. %	0	0	0.5	0	0		0.5			
			M.C.T.	1.8	1.9	2.0	1.8	1.8		2.0			
			Sph. I.	0.24	0.26	0.26	0.25	0.25		0.28			
64	20 % saponin (titer 1:1 536) +	0.1 ml. (256 H.U.)	R.B.C.	3.97	3.61	4.66	4.33	4.05		3.89		4.41	
			M.C.V.	103	112	84	89	86		87			
			M.C.D.	7.58	7.38	7.17	7.10	7.36		7.42			
	bean extract IV (titer 1:3 072)	0.1 ml.	Micr. %	0	0	1.5	1.0	0		0			
			M.C.T.	2.3	2.6	2.1	2.2	2.0		2.0			
			Sph. I.	0.30	0.35	0.29	0.31	0.27		0.27			
131	bean extract V (titer 1:3 072) +	0.1 ml.	R.B.C.	4.95	5.58	5.13	4.84	4.94	4.71	5.04			
			M.C.V.	80	74	75	79	80		77			
			M.C.D.	7.09	6.87	6.92	6.94	6.94		7.10			
	streptolysin IV (titer 1:192)	0.5 ml. (160 H.U.)	Micr. %	1	1	1	1.5	1		0			
			M.C.T.	2.0	2.0	2.0	2.1	2.1		1.9			
			Sph. I.	0.28	0.29	0.29	0.30	0.30		0.27			
132	streptolysin IV (titer 1:192) +	0.5 ml. (160 H.U.)	R.B.C.	4.96	5.63	3.82	4.60	3.92	4.15	4.31			
			M.C.V.	81	76	88	84	89		79			
			M.C.D.	7.13	7.12	6.99	6.89	6.84		6.82			
	bean extract V (titer 1:3 072)	0.1 ml.	Micr. %	0.5	0.5	2.	1	0.5		1			
			M.C.T.	2.0	1.9	2.3	2.3	2.4		2.1			
			Sph. I.	0.28	0.27	0.33	0.33			0.31			

the erythrocyte count. At the same time the spherical index of the erythrocytes increased somewhat in all the experiments.

Intravenous injection of streptolysin was followed by immediate hemolysis with hemoglobinuria and a relatively insignificant decrease in the erythrocyte count. No distinct spherocytosis was observed in the majority of these experiments. Repeated intraperitoneal injections of streptolysin caused no hemolysis in guinea-pigs.

Bean extract with a high agglutinin titer injected intravenously or intraperitoneally into guinea-pigs did not cause a definite hemolysis in vivo, and hemagglutination was not found in the blood.

The hemolytic effect in vivo of saponin or streptolysin in combination with bean extract was not stronger than that of saponin or streptolysin alone.

CHAPTER VII

EXPERIMENTS IN VITRO

TITRATIONS

The effect of hemolysins in the living organism has been described in the preceding two chapters. To compare this effect with that in vitro, the titers obtained by one method only will not suffice. In titration in vitro the hemolysins act on the blood cells under conditions greatly differing from those after injection into the living animal.

In all the routine titration methods, a suspension of not more than 5 per cent blood cells is generally used. In the one employed by Dameshek and Schwartz (1938) and in this work, the suspension contained 2 per cent of blood cells. But in each test-tube there was only 0.5 ml. of this suspension and 2.5 ml. of other elements, hence the influence of the hemolysins on a blood cell suspension, of which the concentration is only $\frac{1}{3}$ per cent, can be determined by this method. In vivo, on the other hand, the volume per cent of blood corpuscles is 40 to 50.

In ordinary titration the amount of normal serum is also very much less than in whole blood, in which it is 50 to 60 per cent, because a very small amount of normal serum has been added as complement. In the method used in this work, 0.5 ml. guinea-pig serum, diluted 1:10, was added which in the total contents of the tube, 3 ml., is only 1:60 or 1.67 per cent. Thus, in the experiments on animals, the immune hemolysin has to influence about 150 times more blood corpuscles per ml. in the presence of 30 times as much normal serum as in the titrations. No serum was added for determination of the titer of the saponin dilutions and the streptolysin.

Several different titrations with varying amounts of corpuscles and normal serum (fresh guinea-pig serum) were done in order to determine the significance of these two factors, i. e. the concentrations of normal serum and of blood cells.

The following series of experiments with immune serum, saponin, and streptolysin were made:

- 1) »Ordinary» titration as described in Chapter III.
- 2) Titration with the same blood cell concentration as in the first series, but a

larger amount of normal serum. In Series 2 a the total amount of 3 ml. per test-tube was retained, but instead of the normal serum dilution being 1:10, it was now only 1:2. In Series 2 b, undiluted serum was used, each tube containing 0.2 ml. hemolysin dilution, 0.3 ml. normal serum, and 0.1 ml. of a 2 per cent blood cell suspension.

- 3) Experiments with higher blood cell concentrations: 0.2 ml. hemolysin dilution, 0.1 ml. fresh guinea-pig serum, 1:10, and 0.3 ml. guinea-pig corpuscles. The complement concentration was thus the same as in Series 1.
- 4) Titration with whole blood. As already stated, the titers given in this work are the final dilutions in the last test tubes with total or visible hemolysis, respectively. To compare these values with those obtained in the first experimental series, they should in preference be multiples of 3, which was considered when the different titrations were worked out. For that reason 0.4 ml. defibrinated guinea-pig blood was added to 0.2 ml. hemolysin dilution (1:2:4:8 etc.) in each tube in the last series. Actually the tubes thus contained $\frac{2}{3}$ blood and not whole blood.

The results and the final amounts of normal serum and blood cells in the test tubes are given in Table 13. When several experiments were made with the same method, the titers varied somewhat but not more than 1:2. The value obtained in the majority of the experiments is given, or if two values had been obtained an equal number of times, the lower value is given. The total hemolysis titer and the visible hemolysis titer are given separately. Difficulties like those in the »ordinary» titrations (Series 1) were sometimes experienced in these experiments in determining which test tube was the last in which hemolysis was visible. A source of error also lies in the fact that in titrations with whole blood or with half the tube containing normal serum or corpuscles, the same amount of fluid (3 ml.) could not be used as in Series 1 because too large amounts of guinea-pig blood would have been required for the purpose. Only 0.6 ml. per tube was used instead. This amount of fluid naturally heated more rapidly in the thermostat than the larger amounts. In Series 2 a, 3 ml. per tube and five times more normal serum than in Series 1 was however used so as to allow the two series to be compared.

Owing to the sources of error in these titrations, very far-reaching conclusions should not be made.

It may, however, be stated that:

1. The titer obtained is greatly dependent on the titration method, i. e. on the proportions between the different ingredients in the test tubes.
2. The presence of normal serum inhibits the saponin and streptolysin hemolysis, while a larger amount of normal serum, e. g. excessive addition of complement, promotes the immune serum hemolysis. Although the smaller amounts of normal serum (complement) in Series 1 sufficed for hemolysis

of all corpuscles in the tubes in which the amboceptor concentration was highest, the titers of immune sera for total and visible hemolysis were much higher in Series 2 a and 2 b in which more normal serum had been used.

In an additional experiment it was shown that the complement in normal serum is responsible for its hemolysis promoting effect. When Series 2 b was repeated, but, inactivated, instead of fresh guinea-pig serum being added, as well as a small amount of fresh guinea-pig serum, diluted 1:10, as complement, in the same proportion as in Series 1, then the titers were much lower than those in Series 2 b and even lower than in Series 1. It was thus found not only that inactivated serum lacks the hemolysis promoting effect of fresh serum, but that it may even inhibit hemolysis.

3. An increased amount of blood cells prevents hemolysis when the hemolysin consists of immune serum, saponin, or streptolysin.
4. When the influence on whole blood is studied by means of titration methods, the results are affected both by a high normal serum content and a high blood cell concentration. The saponin titer is then lower than in »ordinary» titration. The streptolysin titer is influenced in the same way, and no total

TABLE 13.
INFLUENCE OF VARIOUS CONCENTRATIONS OF NORMAL SERUM
AND BLOOD CELLS ON THE HEMOLYSIN TITER

	1		2 a		2 b		3		4	
	»Ordinary» titration (method in Chapt. III)		Titration with an excess of normal serum		Titration with a large excess of normal serum		Titration with 50 % red cells		Titration with who blood	
Total amount per test tube	3 ml.		3 ml.		0.6 ml.		0.6 ml.		0.6 ml.	
Concentration of normal serum	1:60		1:12		1:2		1:60		ca. 1:3	
Concentration of corpuscles	1:300		1:300		1:300		1:2		ca. 1:3	
	Titer		Titer		Titer		Titer		Titer	
	Total hemolysis	Visible hemolysis	Total hemolysis	Visible hemolysis	Total hemolysis	Visible hemolysis	Total hemolysis	Visible hemolysis	Total hemolysis	Visible hemolysis
Saponin 20 %	1:1 536	1:6 144			1: 384	1: 1 536	1:24	1:768	1:24	1:3
Streptolysin 0	1: 192	1: 384			1: 12	1: 192	-($<1:6$)	1:192	-($<1:6$)	1:3
Serum II	1: 48	1:1 536	1: 768	1:3 072	1:1 536	1: 6 144	-($<1:6$)	1:384	-($<1:3$)	1:3
Serum IV	1: 24	1: 384	1: 96	1: 768	1: 96	1: 768	-($<1:6$)	1:192	-($<1:3$)	1:3
Serum V	1: 192	1:3 072	1:1 536	1:6 144	1:3 072	1:12 288	-($<1:6$)	1:768	-($<1:3$)	1:3

hemolysis was obtained in these experiments with streptolysin and whole blood. The titer of immune sera was influenced so that no total hemolysis was obtained, while the visible hemolysis titer was higher, on the other hand, than in »ordinary» titration. In these experiments, total hemolysis of whole blood was caused only with saponin solution.

The agglutinin titer was also read in these titrations. The results of the various experiments were, however, quite contradictory, hence no reliable conclusions can be drawn. In general, the agglutinin titer seemed to fall with increased normal serum concentration and with increased blood cell concentration. However, in some tests a high serum concentration seemed to promote agglutination. This may have been due to the presence of incomplete antibodies in the immune sera used. As this question was not studied closely and the results were contradictory, as already stated, the values for the agglutinin titer are not included in Table 13. The greatest interest in the problem treated in this work concerns the agglutination in whole blood. In all the tests with immune serum it was much weaker than in »ordinary» titration and also if bean extract (See Table 14) was used. No hemolytic effect was obtained with bean extract in the different titrations, nor did streptolysin or saponin agglutinate blood cells.

EXPERIMENTS WITH WHOLE BLOOD IN VITRO

Among these titrations the experiments with whole blood are naturally of primary interest for comparison with the experiments on animals. But, even if whole blood is used for titration, the conditions differ from those which influence the action of hemolysins in vivo.

The results of these titrations were read for the first time after two hours, whereas, in the experiments on animals, blood samples were taken after three hours, one day, two days, etc. The specific hemolysis in the test-tubes is, however, considered complete within two hours either with the use of immune serum (Sachs 1930) or streptolysin (Kalak 1942), and the titer was actually not changed if the

TABLE 14.
AGGLUTININ TITER IN »ORDINARY» TITRATION AND IN TITRATION
WITH WHOLE BLOOD

	»Ordinary» titration	Titration with whole blood
Serum II	1:3 072	1: 96
Serum IV	1:1 536	1: 12
Serum V	1:6 144	1:192
Bean extract	1:3 072	1: 96

tubes were placed in the thermostat for a further two or four hours after reading. Regarding saponin, P o n d e r's (1943) »time-dilution curves« show that the hemolysis is not quite, but almost complete after two hours. When the saponin dilutions were titrated, the results were read after two and six hours. It was generally observed that the intensity of the hemolysis in the last test-tube with visible hemolysis had increased somewhat in the four hours between the two readings, but the titer had not changed.

The temperature is a differing factor. In the titrations it was 37°C. whereas the internal body temperature of the guinea-pig is about 38.5°C. (O p p e n h e i m e r and P i n c u s s e n 1925) — but this is probably of no particular importance.

But, the fact is no doubt of great significance that, in the titrations, the corpuscles are allowed to sink, whereas, in vivo, the cells are constantly moving in the blood plasma.

Another factor of importance is the measure used for hemolysis in vivo and in vitro. In the first case blood counts are concerned and in the second titers.

In vivo there are further differences which are difficult or impossible to assess, e. g. the relation between anti-bodies and other cells than the red ones, the oxidation and reduction of hemoglobin, other metabolic processes and the influence of various organs on the circulating red cells, etc.

It is impossible to reproduce in vitro all the known and unknown factors which act on the blood in vivo. However, in order to be able to compare the hemolysis in vivo with something more similar than the titrations, a few additional experiments with whole blood were made in vitro. In these, sedimentation of the cells was prevented and the hemolysis assessed by means of blood counts. Glass-tubes, taking about 2.5 ml. and furnished with closely fitting rubber stops, without »dead angles«, were used for the purpose. The tubes were filled under aseptic conditions with 2 ml. of defibrinated guinea-pig blood and a small amount of hemolysin. They were then placed in a thermostat, the temperature being kept between 37.8 and 38.8°C. They were fixed on a disk which rotated six times a minute round a horizontal axis, the longitudinal axis of the tubes forming an angle of about 20° with the rotation axis of the disk.

Besides the usual tests, a sample was taken from the tubes and put into a capillary tube, inner diameter 0.8 mm., and its openings closed with a rubber ring. The tubes were then centrifuged in order to observe macroscopically whether hemolysis occurred in the serum separated.

Since these experiments were made with a view to compare the results with the effect of hemolysins in vivo, the approximate concentration of the substances in question in the blood of the experimental animals had to be found. The blood volume was determined in 11 guinea-pigs by the method described in Chapter III. The amount of blood varied between $\frac{1}{11}$ and $\frac{1}{15}$ of the body-weight, average $\frac{1}{13.5}$, i. e. 7.4 per cent of the body-weight. As the doses in the animal experiments

were calculated per 100 gm. body-weight, the hemolysin doses used had to act on about 7.4 ml. of blood. The hemolysin concentration in the experiments with whole blood *in vitro* was accordingly calculated to correspond with the concentration *in vivo*.

Some experiments were first made with the different hemolysins in order to test the effect of the various doses on the number of blood cells. Samples for erythrocyte count only and for macroscopical statement of hemolysis were taken. The initial value was determined only from the control tube to which, instead of hemolysin, the same amount of saline had been added. Two samples were taken and the mean assumed to be the initial value. Before filling a series of tubes, the blood was shaken carefully for 15 minutes so that all tubes should contain the same number of erythrocytes.

The results are shown in Table 15. For comparison with experiments on animals (Tables 6—12) the doses which correspond to the different hemolysin concentrations are given in H. U. per 7.4 ml. of blood (100 gm. body-weight). Samples were taken from the tubes after three, six and 24 hours. The control tubes also showed more or less distinct hemolysis 24 hours after starting the experiment, but never after six hours. Hence the fall in the blood count after six hours may be considered specific, but the value after 24 hours can hardly be so. There is, however, a possibility that the erythrocytes which have been influenced, but not dissolved, by the hemolysin, are more easily affected by mechanical trauma in the rotating tubes and by the non-physiological conditions, and that some of these corpuscles were dissolved already within six hours, although this did not occur in the control tubes. As a rule the erythrocyte count still decreased during the second three-hour period but much less than in the first three hours.

Comparing the fall in the erythrocyte count in whole blood *in vitro* in six hours with the total decrease in the experiments on animals we see that streptolysin dissolved more blood cells *in vitro*, and also saponin, except in two animals. Immune serum, on the other hand, dissolved a much larger number of erythrocytes *in vivo* than *in vitro* under the conditions now mentioned. Table 15 shows furthermore that the hemolytic effect of immune sera was terminated after six hours, since the erythrocyte count did not decrease more than in the control tubes in the following 18 hours. But it still dropped during the last period of the experiments in the tubes which contained saponin or streptolysin. As already stated, it is doubtful whether this hemolysis can still be considered specific. These experiments verify the incongruity, observed previously (Chapter V), in the effect of the different sera *in vivo* and *in vitro*. Of the sera used, No. V had the strongest hemolyzing effect *in vivo* but *in vitro* it caused only a rather insignificant hemolysis when the concentration was the same as in the animal experiments. The other two sera, whose action, with the respective doses, was somewhat weaker than that of serum V *in vivo*, were much stronger than serum V *in vitro*, yet less effective than *in vivo*.

TABLE 15.

CHANGES IN RED BLOOD COUNT IN EXPERIMENTS WITH WHOLE BLOOD IN VITRO

Contents of test tubes	Corresponding dose in H.U./7.4 ml. blood (100 gm. body-weight)	Initial value		After 3 hours		After 6 hours		After 24 hours	
		R.B.C.	Lysis	R.B.C.	Lysis	R.B.C.	Lysis	R.B.C.	Lysis
2 ml. blood + 0.27 ml. saline	—	4.90	—	4.81	—	4.88	—	4.64	(+)
» + 0.27 ml. streptolysin (titer 1:192)	320	»		2.70	+	1.88		1.60	
» + 0.27 ml. » , dil. 3:4	240	»		3.33	+	2.80		2.17	
» + 0.27 ml. » , dil. 1:2	160	»		3.43	+	3.20		2.64	
2 ml. blood + 0.08 ml. saline	—	4.65		4.62	—	4.68	—	4.61	—
» + 0.08 ml. saponin solution	768	»		2.38	+	1.84		1.32	
» + 0.08 ml. » » , dil. 2:3	512	»		3.15	+	2.93		2.53	
» + 0.08 ml. » » , dil. 1:3	256	»		4.03	+	3.89		3.36	
2 ml. blood + 0.08 ml. saline	—	5.76	—	5.67	—	5.74	—	5.24	+
» + 0.08 ml. ser. II, dil. 1:2	12	»		3.83	+	3.76		3.86	
» + 0.08 ml. ser. II, dil. 1:4	6	»		4.71	+	4.08		3.83	
» + 0.08 ml. ser. V, dil. 1:24	4	»		4.97	+	4.84		4.39	
» + 0.08 ml. ser. V, dil. 1:48	2	»		5.36	+	5.39		4.91	
2 ml. blood + 0.08 ml. saline	—	4.79	—	4.79	—	4.88	—	4.64	(+)
» + 0.08 ml. ser. IV, dil. 1:2	6	»		4.60		4.37	(+)	4.33	(+)
» + 0.08 ml. ser. IV, dil. 1:4	3	»		4.71		4.57	±	4.34	(+)

The very mild effect of immune serum amboceptor in vitro may be thought to be due to the lack of complement. In order to solve this question, an experiment was made in the following manner: two tubes were filled with 2 ml. of blood and a certain amount of serum II, two tubes with blood and serum V, and one control tube with blood and saline. They were then rotated slowly in the thermostat in the usual way and samples were taken for erythrocyte count after three hours. All the tubes were then slowly centrifuged for two minutes, and 0.75 ml. of serum was replaced by fresh guinea-pig blood in one of the tubes with immune serum II and in one with immune serum V. The hemolysis in these tubes was not increased in comparison with those in which the serum had not been exchanged.

The immune serum concentrations in the experiments corresponded to the largest doses used in the animal experiments. As described in Chapter V, intense agglutination of the animals' red cells always occurred after these doses. Hence it seems rather surprising that no agglutination of cells was observed in the experiments now described with immune serum in vitro. But this agrees with the results of the agglutinin titrations with whole blood (Table 14). Agglutination occurred only in

TABLE 16.

CHANGES IN SHAPE OF RED BLOOD CORPUSCLES IN EXPERIMENTS WITH WHOLE BLOOD IN VITRO

Contents of test tubes	Corresponding dose in H.U./7.4 ml. blood		Initial value	After 3 h	After 6 h
2 ml. blood + 0.27 ml. saline	—	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis	4.54 84 7.09 0 2.1 0.30	4.67 81 7.16 0.5 2.0 0.28	4.68 81 7.03 0.5 2.1 0.30 —
2 ml. blood + 0.27 ml. streptolysin (titer 1:192), diluted 1:2	160	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis		4.29 87 7.16 0 2.2 0.31	3.96 95 7.17 0 2.4 0.33 +
2 ml. blood + 0.08 ml. saline	—	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis	5.28 82 7.22 0 2.0 0.28	5.20 84 7.16 0 2.1 0.29	5.23 84 6.96 1.5 2.2 0.32 —
2 ml. blood + 0.08 ml. ser. II, dil. 1:4	6	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis		4.30 91 7.07 1.5 2.3 0.33	3.69 104 7.15 0 2.6 0.36 +
2 ml. blood + 0.08 ml. ser. V, dil. 1:48 ₁	2	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis		5.04 84 7.09 0 2.1 0.30	4.96 83 7.19 0.5 2.0 0.28 +
2 ml. blood + 0.08 ml. 20 % saponin solu- tion, diluted 1:3	256	R.B.C. M.C.V. M.C.D. Micr.% M.C.T. Sph. I. Macroscopic hemolysis		4.38 87 6.22 8 2.9 0.47	4.16 89 6.26 6 2.9 0.46 +

the tubes with higher immune serum concentrations than in these experiments. This question will be discussed more closely in Chapter VIII.

Non-specific hemolysis occurring after 24 hours in the experiments just mentioned, further trials with whole blood *in vitro* were limited to six hours. These trials concerned spherocytosis, and samples were taken for red cell counts and determination of their volume per cent and diameter. Experiments were made with only one concentration of each substance, i. e. streptolysin, saponin, serum II and serum V. The results are given in Table 16. It was found that saponin caused very pronounced spherocytosis while serum II and streptolysin caused slight spherocytosis, and serum V none at all. The concentrations of saponin and streptolysin were equal to a small dose, and those of the two sera equal to a large dose *in vivo*.

SUMMARY OF CHAPTER VII

Higher titers for immune serum were obtained in hemolysin titrations by various methods when the amount of normal serum was increased, i. e. when complement was added in excess, but normal serum inhibited the hemolytic effect of saponin and streptolysin. When the blood cell concentration was increased, the titers for immune serum, saponin, and streptolysin were lower. With the use of whole blood, the saponin and streptolysin titers were much lower in these titrations than in »ordinary» titration. Immune sera did not cause total hemolysis of whole blood, but the visible hemolysis titer was higher than in »ordinary» titration. With immune sera and bean extract, the agglutination of whole blood was weaker than that of a diluted blood cell suspension.

In experiments with whole blood in slowly rotating tubes, the decrease in the erythrocyte count caused by saponin and streptolysin in six hours was greater than the red cell decrease with the same blood cell concentration in the experiments on animals. But the hemolyzing effect of immune sera was weaker *in vitro* than *in vivo*, and not strong enough to decrease the blood cell count nearly so much *in vitro* as in the animal experiments when the specific immune serum concentration was equal in both instances. The action of the different sera on whole blood *in vitro* was not proportional to that *in vivo*.

Agglutination of blood cells did not occur in experiments with immune serum and whole blood in motion, although the same immune serum concentration in the blood of experimental animals always caused intense agglutination of their red cells.

Saponin induced marked spherocytosis *in vitro*, whereas streptolysin and one of the immune sera caused slight spherocytosis and another immune serum none at all.

CHAPTER VIII

COMMENTS

THE ACTION OF IMMUNE SERUM IN VIVO AND IN VITRO COMPARED WITH THAT OF SAPONIN AND STREPTOLYSIN

In the preceding chapters, experiments with three different hemolysins have been described, i. e. immune serum, saponin, and streptolysin O. These three were chosen because they represent widely differing categories of hemolysins. While the saponins are fairly well-known substances of the plant world, streptolysin is a bacterial product which has become of great clinical interest owing to its antigenic properties. These two have one characteristic in common — ability to dissolve red blood cells without the addition of other substances. Immune serum has the capacity to agglutinate or to dissolve erythrocytes, but to dissolve only when combined with another substance, i. e. »complement», which is found in fresh normal serum. Hence the immune serum is termed »amboceptor» or »sensitizer».

Also in the experiments on animals the immune serum differed from the other two hemolysins. One single intravenous injection of immune serum induced typical hemolytic anemia which was followed by spherocytosis. Large doses of saponin solution or streptolysin given intravenously did certainly cause immediate intravascular hemolysis, but true anemia did not follow in these cases.

If the quantitative effect of different hemolysins in vivo is to be compared, the doses must be comparable with each other in some way. Hence the strength of the various hemolysins must be given and this is generally expressed by a titer; this is the highest dilution in which the substance in question can dissolve a blood cell suspension under certain conditions. With this procedure in the experiments described here, and giving the doses in hemolytic units (H. U.), which is a function of the titer, it will be seen that *the effect of immune serum is very much stronger per H. U. in vivo than that of saponin and streptolysin.* In the animal experiments the doses varied between 0.5 and 12 H. U. per 100 gm. body-weight, while saponin was given in doses of 256 to 819 H. U., and streptolysin in doses of 120 to 240 H. U. per 100 gm., yet a much greater decrease in the erythrocyte count was obtained with immune serum than with the latter two substances. The quantitative effect of the different sera varied somewhat. Serum V was the most effective since 4 H. U. per 100 gm. induced fatal anemia. Reckoning with regeneration of erythrocytes, it may be

stated that 4 H. U. serum V per 100 gm. dissolved all the blood cells of the animal, but naturally it died before the erythrocyte count had dropped to nil. Even 2.4 H. U. of this serum had the same effect, and one animal died of as little as 2 H. U. All the animals given 6 H. U. serum II per 100 gm. survived although severe anemia developed. 12 H. U. of this serum sufficed to dissolve all the animal's blood cells. It seemed as if the action of serum V was about three times stronger than that of serum II in vivo, but this is not quite definite as the methodical error is 50 to 100 per cent (Boyd 1947) in serological titrations. The difference between the effect of immune sera in vivo, on the one hand, and on the other saponin and streptolysin, was of an entirely different order of magnitude since, for instance, 6 H. U. serum II or 2 H. U. serum V was much stronger in vivo than the largest doses of saponin and streptolysin, yet they were expressed in hundreds of H. U.

The action of the different hemolysins in vivo and in vitro will here be compared more closely than in Chapter VII and, for simplicity, the doses will be given in H. U. as before. The blood volume of the guinea-pig averages about 7.4 ml. per 100 gm. and its hematocrit value 45. Since 1 H. U. can dissolve 0.1 ml. of a 2 per cent erythrocyte suspension, $74 \times \frac{45}{2} = 1665$ H. U. per 100 gm. would be required in order to dissolve all the animal's corpuscles. As the blood count averages 5.41 millions per cu. mm., the dose required to decrease the number of erythrocytes to 1 million should be about 300 H. U. per 100 gm. body-weight. The decrease in the red cell count was small and varied greatly in the experiments with saponin and streptolysin, hence it is impossible to state precisely how many corpuscles a certain amount of these substances can dissolve in vivo. It seems, however, as if the dose required to decrease the erythrocyte count, for instance by 1 million, would be of about the same size as the one obtained in the previous rough calculation. For instance, the saponin dose 512 H. U. per 100 gm. decreased the erythrocyte count by 1.4 millions on an average in four experiments (See Table 9). The streptolysin doses in these experiments, so few in number, were, expressed in H. U., two or three times smaller than the saponin doses, and the resulting drop in the erythrocyte count was also less. The action of the anti-erythrocyte serum was, contrary to these two hemolysins, one hundred times stronger in vivo than calculated. The dose 6 H. U. serum II per 100 gm. hemolyzed on an average 3.5 million corpuscles per cu. mm. in four non-splenectomized animals (Table 6). Thus 2 H. U. should suffice to dissolve 1 million erythrocytes per cu. mm., and yet the effect of serum II was about three times less in vivo than that of serum V. To judge from a few experiments, the action of serum IV was almost similar to that of serum II.

The figures given cannot be considered exact, yet they express the order of magnitude of the hemolytic effect of the anti-erythrocyte sera in vivo, and it was observed that *the effect of two different sera was more than one hundred times stronger and that of a third serum several hundred times stronger in vivo than in vitro. The effect of sera*

in titration in vitro was, however, used as the standard. The hemolytic action on a diluted blood cell suspension, with a small amount of normal serum as complement, was tested by this titration method, as commonly done. In some other titrations it was found that higher blood cell concentrations gave lower titers. In fact, the number of blood cells *in vivo* and *in vitro* was taken into account in the calculation, although it is not known whether the relation to the blood cell count is as quantitative as assumed. In titration by the »ordinary» method, the amount of complement sufficed for total hemolysis, it is true, when the amboceptor concentration was high, but it was not optimal. Amboceptor and complement can, as shown by Eagle (1937), replace one another within certain limits, and various titrations with low erythrocyte concentration proved that an increased amount of normal serum raised the immune serum hemolysin titer, when half of the test-tube contained normal serum, to a titre that was four to 32 times higher (Table 13) than in »ordinary» titration. *Hence it is quite probable that the large supply of complement in the blood of the living animal plays an important role in the unexpectedly strong hemolysis in vivo. But this factor alone is certainly not the whole explanation.* In the experiments with 50 per cent normal serum and only $\frac{1}{3}$ per cent blood cell concentration, the complement surplus was much greater in relation to the number of blood cells than *in vivo*. In the living organism there is another factor which cannot be evaluated, i. e. the regeneration of the complement. It must, however, be very rapid in order to increase the surplus more than in this titration, within a reasonable time. Even if the immune serum titers are raised four to 32 times and the doses are calculated in H. U. on the basis of the new values according to the titration plan with a large surplus of complement, the fact still remains that the action of immune serum is much stronger *in vivo* than *in vitro*. In titration with whole blood, none of the immune sera induced total hemolysis, not even in the dilution 1:3 which is a concentration that was not nearly reached in the animal experiments. Normal serum inhibited the hemolytic effect of saponin and streptolysin, yet saponin, diluted 1:24, caused total hemolysis of whole blood.

Further experiments were made in vitro with whole blood in motion. The hemolysis was assessed by means of blood cell counts. Under these conditions saponin and streptolysin dissolved a somewhat larger number of cells than in vivo, whereas none of the three immune sera, in the same concentration, dissolved nearly as great amounts of blood cells as in the living animal. Adding complement did not increase the hemolytic effect of the immune sera. This speaks against the assumption that regeneration of complement is the most important factor among the causes of the strong hemolytic effect of immune sera after injection into an animal. Also in these experiments it was noticed that the effect of the various sera *in vivo* was not proportional to that *in vitro*.

To judge from the titrations, the hemolysis *in vitro* is terminated after two hours, as described in Chapter VII. Experiments with whole blood in rotating tubes showed that the hemolysis was strongest during the first three hours but continued slowly in the majority during the following three hours. After six hours from the

beginning of the experiment, it had stopped, however, when immune sera were used, whereas it is not quite certain whether the saponin and streptolysin hemolysis was terminated after six hours.

The time taken by the hemolysis in the experimental animal was quite different. In the first three hours after an injection of hemolytic immune serum it was relatively insignificant but the erythrocyte count decreased during three to five days, mostly during the second day. After injection of saponin or streptolysin the erythrocyte count did not drop sufficiently to justify any definite opinion about the course of the hemolysis, but in any case it was not progressive in character as after injection of immune sera into an animal.

It was thus found that anti-erythrocyte serum dissolves much larger amounts of erythrocytes in vivo than in vitro, whether this hemolysis in vivo is compared with that in different titrations or with experiments with whole blood in motion in vitro. Furthermore, the hemolysis lasts much longer in vivo after injection of immune serum than in vitro and it may be concluded that: In experimental hemolytic anemia, the hemolysis in vivo is not mainly caused by the direct effect of the immune hemolysins on the red blood cells — it is essentially due to the living organism taking an active part in the destruction of erythrocytes.

On the other hand, the hemolysis which occurs in the experimental animals after injection of saponin or streptolysin is mainly the direct cause of the action of these substances on the red blood cells. Comparing hemolysis induced by these substances in vivo and in vitro, it will be found that it is equally strong in vitro and in vivo or stronger in vitro, depending on how the hemolysis in vitro has been assessed quantitatively. Besides, in these cases the hemolysis in vivo has not the lengthy, progressive course as after injection of immune serum.

THE ROLE OF THE ORGANISM IN THE HEMOLYSIS IN EXPERIMENTAL HEMOLYTIC ANEMIA

What is there in the organism that produces a destructive effect on erythrocytes after injection of anti-erythrocyte serum? A factor which may be of some importance for the hemolysis in vivo is the large supply and the regeneration of complement in the living organism. But it alone, as already stated, will not suffice to explain the whole difference between the hemolysis in vivo and in vitro. That the supply of complement cannot in this respect be of decisive importance is also shown by Young, Ervin and Yuile (1949) in their experiments on dogs which have already been mentioned. They studied the complement content of the blood of these animals after injection of anti-erythrocyte serum and found a moderate decrease during the first hours only. Neither these authors nor Dameshek and Schwartz (1940) observed free antibodies in the test animals' blood, hence there can be no question of an activation of the antibodies in vivo.

Other possibilities must be considered and attention will be paid first to the spleen. Some parallel experiments on normal and splenectomized animals were

described in Chapter V. In these the hemolysis was nearly always stronger in the animals with an intact spleen. The material was not very large, but since the same results were obtained in all the experiments, except one, and also in B a n t i's (1913) and my own previous work (W a s a s t j e r n a 1948 a), it may be concluded that *the spleen plays an important part in the hemolytic activity of the living organism in experimental hemolytic anemia*. No importance can be attached to the fact that J o a n n o v i c s (1904) and F i l o (1936) as well as T i s c h e n d o r f and F r a n k e arrived at opposite results since no direct quantitative comparison of the erythrocyte destruction in normal and splenectomized animals were made in their investigations. In the experiments described in Chapter V, the splenectomized animals acquired hemolytic anemia of the same type as the others but less severe. The hemolysis was also more intense in the splenectomized animals than that induced in vitro by immune serum of the same concentration. This shows that the living organism takes part in the erythrocyte destruction in experimental hemolytic anemia even if the spleen has been removed. This investigation does not reveal what other organs have a hemolytic effect, similar to that of the spleen, but it is very probable that the other reticuloendothelial organs have the same capacity. As the splenectomized animals' anemia differed only quantitatively, it may be thought that the hemolyzing effect of the living organism is generally of the same kind as that of the spleen. The mechanism active in the spleen that produces the destructive effect on erythrocytes in experimental hemolytic anemia will therefore be discussed.

E m e r s o n, S h e n and C a s t l e (1946) and Y o u n g (1947) have shown that the spleen can retain spherocytes, selectively, from the circulating blood. W h i p p l e (1940), contrary to K n i s e l y (1936), considers that the blood circulation in the spleen is »open», and that the spherocytes cannot pass as easily as normal erythrocytes through the openings of the venous sinuses; hence they remain in the pulp where they are destroyed. In the present material, an increased number of microcytes, which probably were also spherocytes, were observed in some splenectomized animals, untreated in other respects (See Chapter IV). This may have been due to the fact that there was no spleen to withdraw spherocytes from the blood, but the fact that the mean spherical index of the erythrocytes of the splenectomized animals was not increased speaks against this assumption. If the lack of a spleen and its destructive effect on the spherocytes explains the action of immune sera being weaker in splenectomized animals, then the spherocytosis would be more marked in these animals than in normal ones after injection of immune serum. The case was, however, quite the contrary, as stated in Chapter V, i. e. the spherocytosis was on the whole proportional to the hemolysis and in consequence more intense in animals with an intact spleen than in those that had had it removed. *Thus, the spherocytosis in experimental hemolytic anemia was at least largely due to the living organism, i. e. the spleen, among other organs*. Also the experiments with whole blood in vitro showed the same results. In these, only saponin induced

considerable spherocytosis, while one of the two immune sera increased very slightly the spherical index of the erythrocytes and the other serum not at all. By influencing the erythrocytes directly, saponin thus induces intense spherocytosis but the living organism counteracts the effect, since the spherocytosis in the animal experiments was not so marked as in the experiments in vitro with the same dilution of saponin. Evidently immune serum affects the blood corpuscles so that the living organism first changes their shape — the cells become more spherical — and then dissolves them. In other words, *the antibodies sensitize the blood cells to the influence of the living organism, and the spherocytosis is an intermediate stage of this influence.*

Some authors, among them Baumgartner (1947), maintain that the action of immune serum in vivo is mainly an opsonic action and that the organism acts by ingesting blood cells which have been sensitized by immune serum. *That phagocytosis of red blood cells occurs in experimental hemolytic anemia is beyond doubt. In this work also, intense erythrophagocytosis was observed in the spleen of the animals treated and very sparse erythrophagocytosis in the blood in the periphery. However, this phagocytosis of the red cells does not explain the spherocytosis as it cannot be thought that a phagocyte would first ingest a cell, then change its shape and finally extrude it. This also excludes the possibility that phagocytosis of cells is the most important mechanism of the organism's erythrocyte destructive effect after injection of anti-erythrocyte serum into animals.*

It should be remembered that the agglutinin titer of the immune sera used was higher than their hemolysin titer, and that intense agglutination of the corpuscles was observed in animals acquiring hemolytic anemia after intravenous injection of immune serum. On these grounds it seems very probable that this hemagglutination is an essential stage in the destruction of erythrocytes in vivo. This view is held also by Ham and Castle (1940 a and b). In experiments with concanavalin A they found that it has the capacity to agglutinate blood cells, not only in vitro, but also in vivo when injected intravenously into experimental animals. The hemagglutination in the blood of the animals caused blood stasis in various organs, especially in the spleen which in its turn caused spherocytosis and hemolysis of blood cells. A plausible explanation of the mechanism active in hemolysis in vivo in experimental hemolytic anemia seems to be that there is, and this is of prime importance, an intravascular agglutination followed by increased blood stasis in the spleen and some other organs. The stasis, in its turn, acts on the erythrocytes by means of two different mechanisms, i. e. formation of lysolecithin in the plasma (Bergenhem and Fåhræus 1936) and a metabolic process in the erythrocytes of the stationary blood (Bergenhem and Fåhræus 1936, Fåhræus 1939, Ham and Castle 1940 a and b). This process, and the lysolecithin affect the erythrocytes in that they first become more spherical and are finally dissolved. The part played by the spleen in this connection is thus explained. Besides, agglutinated erythrocytes are more susceptible to mechanical trauma than the free erythrocytes (Shen, Castle and Fleming 1944,

Heilmeyer 1950) and, according to Knisely and co-workers (1947), they are readily ingested by reticuloendothelial cells in the spleen or other organs. The erythrocyte membranes may be injured when antibodies are attached to the cells owing to which the erythrocytes are more easily destroyed through stasis and mechanical trauma.

It is thus very probable that hemagglutination is of great importance in the erythrocyte destruction in vivo, but how the intravascular hemagglutination arises has not so far been made clear. In the experiments described in Chapter VII, it was observed that *although a certain dose of immune serum caused intense agglutination of blood cells in vivo, the same serum in equal dilutions did not cause agglutination in whole blood in the experiments with rotating tubes in vitro*. The titrations showed a somewhat variable effect with the different sera. Serum II, 1:96, and serum V, 1:192, agglutinated whole blood (See Table 14). The former was generally diluted twice before injection and the latter 16 or 32 times. The doses were 0.1 to 0.15 ml. per 100 gm. Calculating with 7.4 ml. of blood per 100 gm., the intravascular dilution of serum II was 1:99, and of serum V 1:1184 or 1:1669 in the majority of the experiments. The serum II dilution in vivo was thus about the same as the agglutination titer in whole blood, but the serum V dilution was higher in vivo than in the last tube with whole blood in which agglutination was observed in the titration. Serum IV, diluted 1:99, caused agglutination in vivo and dilution 1:12 in titration with whole blood. At least the latter two sera thus agglutinated whole blood more readily in vivo than in vitro. These experiments do not explain this difference, but it may be thought that, after the antibodies in vivo have united with the blood corpuscles, yet another substance produced by the living organism and causing agglutination may be attached to the cells. This might be a substance with the same effect as »x-protein» (Wiener 1946 and 1947) which is required for incomplete antibodies to agglutinate blood cells and, in the living organism there may be, owing to constant regeneration, a larger supply of this substance than in whole blood in vitro. These are mere postulations, however. But it seems quite clear that *the living organism increases the agglutinating effect of the immune sera in vivo, and it is probable that the intravascular agglutination is of great pathogenetic significance for erythrocyte destruction in vivo in experimental hemolytic anemia*.

The action of bean extract differed from that of immune serum in respect of hemagglutination in vivo and in vitro. Its titer for agglutination of whole blood was 1:96 and it was used in undiluted form in the animal experiments. When injected intravenously in doses 0.1 to 0.3 ml. per 100 gm., i. e. 7.4 ml. of blood, the dilution in the blood of the animals was only 1:74 to 1:24, but no agglutination was observed in blood samples taken from these animals. Bean extract has the capacity to agglutinate blood cells also in whole blood, but this is counteracted by the living organism, whereas the agglutinating capacity of the immune sera is facilitated, as already stated. Bean extract thus lacks the capacity to sensitize blood cells to agglutination in vivo. To judge from Ham and Castle's (1940 a and b)

and D a m e s h e k and M i l l e r's (1943) experiments on animals, a substance with intense agglutinating action, e. g. pure concanavalin A, can overcome the organism's inhibiting effect on non-specific agglutinins and first cause agglutination and then hemolysis of blood cells *in vivo*. Unfortunately these authors do not give the doses of concanavalin A administered in their experiments. D a m e s h e k and M i l l e r give the agglutinin titer 1:10 000 for the dilution used which is much higher than the bean extract titer in this investigation.

In summarizing it may be said that *relatively small doses of anti-erythrocyte serum can sensitize blood cells to agglutination and hemolysis in vivo, whereas both saponin and streptolysin O, which in vitro have a marked hemolyzing effect, lack this capacity, similarly bean extract which in vitro has an intense agglutinating effect on blood cells.*

EXPERIMENTAL HEMOLYTIC ANEMIA COMPARED WITH HEMOLYTIC ANEMIA IN MAN

D a m e s h e k and S c h w a r t z (1938) have shown that there are great similarities in experimental hemolytic anemia and acquired hemolytic jaundice (acute and subacute hemolytic anemia) in man. In the latter, antibodies are often found in the form of hemolysins or agglutinins, but more often as agglutinins. Even if free antibodies are not found in the serum, the sensitization of blood cells can frequently be demonstrated by C o o m b s' test (C o o m b s, M o u r a n t and R a c e 1945, B o o r m a n, D o d d and L o u t i t 1946, L o u t i t and M o l l i s o n 1946). This agrees with the observations in the animal experiments described above, i. e. that small amounts of antibodies can sensitize blood cells to agglutination and destruction *in vivo*, even if the antibodies are so weak that they cannot, in the same concentration, induce hemagglutination or hemolysis *in vitro*.

In order to find an explanation of the hemolysis in cases in which only agglutinins or sensitization of the blood cells, but no hemolysins, are found, the influence of a »co-hemolysin» in the organism may be considered (L o u t i t and M o l l i s o n 1946, v. S c h u l t h e s s 1949). If the mechanism is the one now assumed to be the most probable in experimental hemolytic anemia, a hypothetical substance such as this is not required to explain the erythrocyte destruction. The mechanism would then be the result of intravascular agglutination accompanied by blood stasis, spherocytosis and hemolysis. There are evidently also other mechanisms which act on sensitized blood cells *in vivo*, i. e. mechanical trauma to agglutinated blood cells in the blood stream, and phagocytosis of sensitized erythrocytes. The agglutinating effect of antibodies is probably promoted *in vivo* by x-protein or similar substances which, as is well-known, enhance the effect of antibodies *in vitro* so that incomplete antibodies agglutinate red blood cells (W i e n e r 1946 and 1947).

The significance of the spleen in acquired hemolytic jaundice varies from case to case. This is best seen from the differences in the effect of splenectomy. In some cases cured by splenectomy the role of the spleen may be that it produces anti-

bodies (Wagley, Shen, Gardner and Castle 1948, Evans and Duane 1949). No conclusion can be drawn on the basis of experimental hemolytic anemia regarding the importance of the spleen for the production of antibodies in acquired hemolytic jaundice. On the other hand, the experiments on animals show that even if the spleen does not produce antibodies, it is important because it takes part in the destruction of blood cells which have been sensitized by antibodies in the blood.

What has now been said about acquired hemolytic jaundice concerns also hemolytic disease of the newborn (erythroblastosis foetalis) to a great extent, and other hemolytic anemias with directly or indirectly demonstrable antibodies in the blood. The mechanism for the destruction of erythrocytes may be the same in these cases as the one now described for acquired hemolytic jaundice. In hemolytic disease of the newborn, Zilliacus (personal communication 1950) found on capillary microscopy that intravascular agglutination actually occurs in the blood of infants with this disease. The hemagglutination is thus not a phenomenon occurring in test-tubes only.

It is difficult to draw any conclusions regarding familial hemolytic jaundice and normal erythrocyte destruction on the basis of the animal experiments in the present investigation. However, these and the experiments in vitro show the dissimilarity in the course of the hemolytic processes in vivo and in vitro. In discussions on hemolytic anemias, it is therefore of great importance to bear in mind that conditions in the living organism and the test-tube are entirely different.

SUMMARY

THE PURPOSE OF THIS INVESTIGATION

The effect of anti-erythrocyte serum on red blood cells of guinea-pigs was studied in vivo and in vitro and compared with that of two other hemolysins, i. e. saponin and streptolysin, and an agglutinin, i. e. bean extract. This was done with a view to comparing how many blood cells a certain amount of the different hemolysins can dissolve in vivo and in vitro, and to studying the alterations in the shape of the erythrocytes and agglutination of red cells in experiments on animals and in vitro. Adult guinea-pigs were used for the purpose. Some of these had been previously splenectomized.

RESULTS

The erythrocyte count in the splenectomized animals was on an average the same as in normal animals. The mean corpuscular volume, mean diameter, mean thickness and spherical index were also the same on an average in animals lacking a spleen and in those with an intact spleen, but in some of the splenectomized animals the number of microcytes in the blood was larger, by comparison with normal animals.

The effect of anti-erythrocyte serum was stronger in vivo than in vitro, that is, a certain amount of serum dissolved a larger number of corpuscles in vivo than in vitro. The results were similar whether the quantitative effect of immune serum in vitro was assessed by different titration methods or whether the decrease in the erythrocyte count in vivo after injection of immune serum was compared with the decrease in the number of erythrocytes per cu. mm. in a test-tube containing whole blood with the same immune serum concentration as in the blood of the experimental animals. The course of the hemolysis in the animal experiments covered a much longer period than in vitro. Parallel experiments with normal and splenectomized animals showed that the decrease in the erythrocyte count was larger in animals with the spleen intact than in the splenectomized animals when the same dose of anti-erythrocyte serum, in relation to the animal's weight, was given.

In titration of immune serum by different methods it was observed that with a high blood cell concentration the hemolysin titer was low, while titration with

an increased amount of normal serum, i. e. excess of complement, gave higher titers than in »ordinary» titration. Yet the excess of complement in vivo can only partly explain the fact that the hemolysis in vivo is stronger than in vitro with the same anti-erythrocyte serum concentration.

Marked spherocytosis occurred in all the animals acquiring hemolytic anemia after injection of anti-erythrocyte serum. It was proportional to the hemolysis and thus more pronounced in normal animals than in splenectomized animals. The spherocytosis, with the same immune serum concentration, was much stronger in the blood of the experimental animals than in whole blood in vitro.

When guinea-pigs were given intravenous or repeated intraperitoneal injections of anti-erythrocyte serum in amounts large enough to induce hemolytic anemia, there was always agglutination of red blood cells in the blood samples. On capillary microscopy it was found that this agglutination was an intravasal phenomenon. The hemagglutinating effect of immune serum was more pronounced in vivo than in vitro. In experiments with whole blood in vitro and the same concentration of immune serum as in the animal experiments there was no agglutination of blood cells.

Saponin and streptolysin, contrary to immune serum, did not dissolve larger numbers of blood cells in vivo than in vitro. They were equally strong in vitro as in vivo, or stronger in vitro, depending on the method used for assessing the hemolysis in vitro. The hemolysis which occurred in the experimental animals' blood after injection of saponin or streptolysin was not of the progressive character observed after injection of immune serum. Comparing the quantitative effect of immune serum in vivo on the one hand, and saponin and streptolysin on the other, it was found that the amount of blood cells which a certain quantity of immune serum can dissolve in vivo, is much larger than the amount of blood cells dissolved by an equal number of hemolytic units of saponin or streptolysin. In the animal experiments, the effect of one hemolytic unit of immune serum was more than one hundred times stronger than that of one hemolytic unit of saponin or streptolysin. The last two substances, in combination with bean extract, did not dissolve more blood corpuscles in vivo than did saponin or streptolysin alone.

Saponin induced marked spherocytosis in experiments with whole blood in vitro and moderate spherocytosis in the experimental animals, while streptolysin, in the doses given, caused only an insignificant spherocytosis in vitro and none at all in vivo in most of the animals.

A strongly agglutinating effect on the red blood cells of guinea-pigs was obtained in vitro with bean extract, but in vivo it did not agglutinate blood cells, although the extract was injected intravenously in doses that should have raised the concentration in the experimental animals' blood to a level which exceeds that in the lowest concentration which, in titration with whole blood, still caused strong agglutination in vitro. The effect of bean extract was not hemolyzing in vitro and probably not in vivo.

CONCLUSIONS

When anti-erythrocyte serum is injected into experimental animals in quantities that will induce hemolytic anemia, then the hemolysis *in vivo* is not directly caused by the effect of the immune hemolysins on the blood cells — it is mainly caused by the action of the living organism on sensitized erythrocytes. The spherocytosis in experimental hemolytic anemia is also mainly the result of the organism's influence on these erythrocytes. Since the agglutinating effect of anti-erythrocyte serum on whole blood is stronger *in vivo* than *in vitro*, the living organism must be partly responsible for the hemagglutination in experimental hemolytic anemia. From this it may be assumed that the organism's hemagglutinating effect is that some substance accumulates on to the erythrocytes whose surfaces are covered with antibodies, and this substance, with the antibodies, agglutinates erythrocytes.

In large doses, the effect of saponin and streptolysin is directly hemolyzing on erythrocytes *in vivo*, but neither these hemolysins nor the agglutinin in the bean extract can sensitize blood cells in a way that enables the living organism to dissolve them.

Erythrocytes covered with immune antibodies are evidently highly susceptible to the organism's erythrocyte destructive effect. The experiments described in Chapter V show that the spleen co-operates in the destruction of these sensitized erythrocytes, but in what manner this organ and other parts of the organism dissolve blood cells cannot be definitely stated. When the erythrocyte membranes are covered with immune antibodies they naturally change in one way or another, but it is not clear whether the change suffices to enable the organism to dissolve the cells or whether they have to be agglutinated. The investigations described in this work cannot definitely answer this question, but the results of the experiments with different hemolysins and agglutinins suggest that intravasal agglutination of the corpuscles very probably contributes actively to increase their sensitivity to hemolysis *in vivo*. When anti-erythrocyte serum was injected into experimental animals, intense hemolysis was regularly accompanied by strong agglutination of the blood cells. On the other hand, agglutination did not occur in the animal experiments with saponin, streptolysin and bean extract, and these three substances did not sensitize blood cells to hemolysis *in vivo*. It is quite probable, to judge from what has been said in Chapter VII, that the action of the organism on agglutinated erythrocytes results in their shapes becoming more spherical, until they finally disintegrate. Phagocytosis of red blood cells is probably also of some importance since it occurs in experimental hemolytic anemia, but the erythrophagocytosis cannot explain the spherocytosis, hence its role cannot be of determining importance for the destruction of erythrocytes.

REFERENCES

- Ashby, W.: *J. Exp. Med.* 29:267, 1919.
- Ashby, W.: *Blood* 3:486, 1948.
- Ask — Upmark, E.: *Svenska läk.-sällsk. förhandl.* 61:197, 1935.
- Banti, G.: *Semaine méd.* 33:313, 1913.
- Baumgartner, W.: *Helv. Med. Acta* 14:502, 1947.
- Belfanti, S. and Carbone, T.: *Gior. Accad. med. Torino* 46:321, 1898.
- Bergenheim, B.: *Acta Path. Microbiol. Scand., Suppl.* 39, 1938.
- Bergenheim, B. and Fåhræus, R.: *Ztschr. f. d. ges. exper. Med.* 97:555, 1936.
- Bessis, M. and Freixa, P.: *Rev. d'hemat.* 2:114, 1947.
- Björkman, S.: *Acta Med. Scand., Suppl.* 191, 1947.
- Boorman, K. E., Dodd, B. E. and Loutit, D. M.: *Lancet* 150:812, 1946.
- Bordet, J.: *Ann. Inst. Pasteur* 12:688, 1898.
- Boyd, W. C.: *Fundamentals of Immunology.* New York 1947.
- Brown, G. M., Hayward, O. C., Powell, E. O. and Witts, L. J.: *J. Path. Bact.* 56:81, 1944.
- Brückman, G. and Wertheimer, E.: *Brit. J. Exp. Path.* 26:217, 1945.
- Callender, T. Sh., Powell, E. O. and Witts, L. J.: *J. Path. Bact.* 57:129, 1945.
- Cantacuzène, J.: *Ann. Inst. Pasteur* 14:378, 1900.
- Castle, W. B. and Daland, G. A.: *Arch. Int. Med.* 60:949, 1937.
- Chauffard, M. A. and Troisier, J.: *Semaine méd.* 28:539, 1908.
- Chauffard, M. A. and Vincent, C.: *Semaine méd.* 29:601, 1909.
- Coombs, R. R. A., Mourant, A. E. and Race, R. R.: *Brit. J. Exper. Path.* 26:255, 1945.
- Dacie, J. V. and Mollison, P. L.: *Lancet* p. 550, 1943.
- Dameshek, W.: *Blood, Special Issue No.* 2:43, 1948.
- Dameshek, W. and Miller, E. B.: *Arch. Int. Med.* 72:1, 1943.
- Dameshek, W. and Schwartz, S.: *Am. J. Med. Sc.* 196:769, 1938.
- Dameshek, W. and Schwartz, S.: *Medicine* 19:231, 1940.
- Donath, J. and Landsteiner, K.: *Ztschr. f. klin. Med.* 58:172, 1906.
- Dudgeon, L. S., Panton, P. N. and Ross, E. A.: *Proc. Roy. Soc. Med. (Path. Sect.)* 2:64, 1909.
- Du Pan, R. M.: *Schweiz. med. Wchnschr.* p. 34, 1948.
- Eagle, H.: *The Laboratory Diagnosis of Syphilis.* St Louis 1937. (Quot. Boyd 1947).
- Emerson, C. P., Shen, S. C. and Castle, W. B.: *J. Clin. Invest.* 25:922, 1946.
- Estren, S. and Dameshek, W.: *Advances in Internal Medicine* 3:45, 1949.
- Evans, R. S. and Duane, R. T.: *Blood* 4:1196, 1949.
- Filo, E.: *Sang* 10:178, 1936.
- Finland, M., Peterson, O. L., Allen, H. E., Samper, B. A. and Barnes, M.: *J. Clin. Invest.* 24:458, 1945. (Quot. Yearbook of General Medicine 1946)
- Freeman, L., Loewy, A. and Johnson, V.: *Am. J. Physiol.* 140:556, 1944.
- Fåhræus, R.: *Nord. Med.* 1:885, 1939.
- Giordano, A. S. and Blum, L. L.: *Am. J. Med. Sc.* 194:311, 1937.

- Gordon, A. S. and Kleinberg, W.: *Proc. Soc. Exp. Biol. Med.* 38:360, 1938.
- Greenwald, H. M.: *Am. J. Med. Sc.* 196:179, 1938.
- Gripwall, E.: *Acta Med. Scand., Suppl.* 96, 1938.
- Haden, R. L.: *Am. J. Med. Sc.* 188:441, 1934.
- Hahn, F. and Lüttgens, W.: *Deutsches Arch. f. klin. Med.* 194:586, 1949.
- Ham, Th. H. and Castle, W. B.: *Tr. A. Am. Phys.* 55:127, 1940 a.
- Ham, Th. H. and Castle, W. B.: *Proc. Am. Phil. Soc.* 82:411, 1940 b.
- Ham, Th. H., Shen, S. C., Fleming, E. M. and Castle, W. B.: *Blood* 3:373, 1948.
- Hedenius, P. and Snellman, B.: *Nord. Med. Tidskr.* 13:914, 1937.
- Heilmeyer, L.: *Deutsches Arch. f. klin. Med.* 179:292, 1936—37.
- Heilmeyer, L.: *Sang* 21:105, 1950.
- Hill, J. M., Haberman, S. and Jones, F.: *Blood, Special Issue No.* 2:80, 1948.
- Isaak, S. and Möckel, K.: *Ztschr. f. klin. Med.* 72:231, 1911.
- Joannovics, G.: *Ztschr. f. Heilkunde* 5:25, 1904.
- Kalbak, K.: *Experimentelle og Kliniske Undersøgelser over O-Streptolysin og Forekomsten af O-Antistreptolysin i Serum.* Copenhagen 1942.
- Knisely, M. H.: *Anat. Rec.* 65:23 and 147, 1936.
- Knisely, M. H., Bloch, E. H., Eliot, Th. S. and Warner, L.: *Science* 106:431, 1947.
- Knisely, M. H., Bloch, E. H. and Warner, L.: *Kong. Dansk. Vidensk. Selsk. Biol. Skr.* 4: No. 7, 1948.
- Kraus, R. and Ludwig, S.: *Wien. klin. Wchnschr.* p. 382, 1902.
- Kraus, R. and Sternberg, C.: *Centralbl. f. Bakt.* 32:903, 1902.
- Lenhartz, H.: *Deutsches Arch. f. klin. Med.* 146:257, 1925.
- Levaditi, C.: *Ann. Inst. Pasteur* 16:233, 1902.
- Levinson, S. A. and Mac Fate, R. P.: *Clinical Laboratory Diagnosis*, Philadelphia 1946.
- Loutit, J. F. and Mollison, P. L.: *J. Path. Bact.* 58:711, 1946.
- Mac Kenzie, D. W., Whipple, A. O. and Wintersteiner, M. P.: *Am. J. Anat.* 68:397, 1941.
- Maegraith, B., Findlay, G. M. and Martin, N. H.: *Nature* 151:252, 1943.
- Mann, F. C., Sheard, Ch., Bollman, J. L. and Balder, E. J.: *Am. J. Physiol.* 74:49 and 497, 1925.
- Meulengracht, E.: *Studier om den Kroniske Hereditære Haemolytiske Ikterus.* Copenhagen 1918.
- Meulengracht, E. and Gram, H. C.: *Hematologisk Teknik.* Copenhagen 1930.
- Mollison, P. L.: *Clin. Sc.* 6:137, 1947.
- Mollison, P. L., Mourant, A. E. and Race, R. R.: *The Rh Blood Groups and their Clinical Effects.* London 1948.
- Muir, R. and Mac Nee, J. W.: *J. Path. Bact.* 16:410, 1911.
- Napier, L. E. and Sen Gupta, P. C.: *Ind. J. M. Research* 31:75, 1943.
- Oppenheimer, C. and Pincussen, L.: *Tabulae Biologicae.* Berlin 1925.
- Ponder, E.: *Proc. Roy. Soc. B* 95:42, 1923.
- Ponder, E.: *J. Gen. Physiol.* 27:483, 1944.
- Ponder, E.: *Hemolysis and Related Phenomena.* New York 1948.
- Ponder, E., Hyman, Ch. and White, L.: *Am. J. Physiol.* 132:18, 1941.
- Race, R. R. and Sanger, R.: *Blood Groups in Man.* Oxford 1950.
- Rich, A. R.: *Physiol. Rev.* 5:182, 1925.
- Rivano, R. and Meneghini, P.: *Pathologica* 39:120, 1947.
- Rous, P.: *Physiol. Rev.* 3:75, 1923.
- Roy, A. C.: *Nature* 155:696, 1945.

- Sachs, H.: Handbuch der pathogenen Mikroorganismen (Edited by Kolle, Kraus and Uhlenhuth) III:279, Vienna 1930.
- v. Schulthess, G.: Acta Haemat. 2:1, 1949.
- Senott, J. S.: Am. J. Dis. Child. 71:269, 1946.
- Shemin, D. and Rittenberg, D.: J. Biol. Chem. 166:627, 1946.
- Shen, S. C., Castle, W. B. and Fleming, E. M.: Science 100:387, 1944.
- Singer, K.: Am. J. M. Sc. 199:466, 1940.
- Singer, K.: Bull. New England M. Center 7:191, 1945 a.
- Singer, K.: J. Lab. Clin. Med. 30:784, 1945 b.
- Singer, K., Miller, E. B. and Dameshek, W.: Am. J. Med. Sc. 202:171, 1941.
- Singer, K. and Weisz, L.: Am. J. M. Sc. 210:301, 1945.
- Sumner, J. B. and Howell, S. F.: J. Bact. 32:227, 1936.
- Tigertt, W. D., Duncan, C. N. and Hight, B. A.: Am. J. M. Sc. 200:173, 1940.
- Tischendorf, W.: Deutsche med. Wchnschr. 74:449, 1949.
- Tischendorf, W. and Franke (Quot. Tischendorf 1949)
- Todd, Ch.: Lancet p. 1663, 1901.
- Todd, E. W.: Brit. J. Exp. Path. 19:26, 1938.
- de Vries, S. J.: Sang 21:278, 1950.
- Wagley, Ph. F., Shen, S. C., Gardner, F. H. and Castle, W. B.: J. Lab. Clin. Med. 33:1197, 1948.
- Wasastjerna, C.: Acta Med. Scand. 132:132, 1948 a.
- Wasastjerna, C.: Nord. Med. 37:113, 1948 b.
- Weld, J. T.: J. Exp. Med. 59:83, 1934.
- Whipple, A. O.: Tr. Coll. Physic. Philadelphia 8:203, 1940.
- Wiener, A. S.: Am. J. Dis. Child. 71:14, 1946.
- Wiener, A. S.: Advances in Internal Medicine 2:439, 1947.
- Wintrobe, M. M.: Clinical Hematology. London 1946.
- Wising, P. J.: Acta Med. Scand. 91:550, 1937.
- Young, L. E.: New York State J. Med. 47:1875, 1947.
- Young, L. E., Ervin, D. M., Christian, R. M. and Davis, R. W.: Science 109:630, 1949.
- Young, L. E., Ervin, D. M. and Yuile, Ch. L.: Blood 4:1218, 1949.
- Zilliacus, H.: Personal communication 1950.

